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A CHEMICAL INVESTIGATION
OF TWO SPECIES OF EUPHORBIACEAE FROM SWAZILAND
AND AN EVALUATION OF COUNTERCURRENT CHROMATOGRAPHY
IN THE FRACTIONATION OF NATURAL PRODUCTS.

Submitted by George W.J. Olivier

for the degree of Doctor of Philosophy

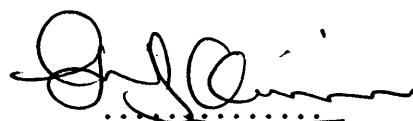
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For Sarah.

".....after watching it for a minute or two, she made it out to be a grin,
and she said to herself 'its the Cheshire Cat.....'"

Lewis Carroll.

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SUMMARY.

The chemical constituents of two species of the plant family Euphorbiaceae from Swaziland were investigated. Four novel diterpene esters were isolated from *Synadenium compactum* N.E. Br. The structures of the compounds were investigated using concerted one- and two-dimensional Nuclear Magnetic Resonance spectroscopic and Mass Spectrometric techniques, and in one instance, X-ray crystallography. The structures of two of the compounds were established with certainty, and were identified as being Δ -12,13, 14-dihydro, 12-deoxy derivatives of the lathyrane type diterpene, incol. The trivial name, synadenol, is given to the new compounds. The structures of the additional two compounds were established as being related to synadenol and possible structures are proposed.

An unusual triterpene, 28-hydroxyfriedelan-3-one, was isolated from *Androstachys johnsonii* Prain. The structure was elucidated by concerted NMR and MS techniques, and confirmed using a single crystal X-ray study.

Countercurrent chromatography was evaluated as a technique for the preparative separation of natural products. Droplet countercurrent chromatography was compared with Ito high speed countercurrent chromatography and modifications to improve the operation of the apparatus are described. A model for the prediction of solute resolution is described and evaluated in the context of its application to the preparative-scale separation of mixtures, using atropine sulphate and hyoscine hydrobromide as model compounds.

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PART ONE.

A CHEMICAL INVESTIGATION OF TWO SPECIES OF EUPHORBIACEAE FROM SWAZILAND.

CHAPTER ONE.

INTRODUCTION.

1.1 The plant family Euphorbiaceae.

1.1.1 Background.

The family Euphorbiaceae, comprising over 300 genera and 5000 species (Willis 1973), is the sixth largest family of flowering plants (Radcliffe-Smith 1986). Although the genera are predominantly tropical in distribution with very few exclusively extratropical, a number, most notably *Euphorbia*, the type genus, are found in sub-tropical and temperate climates (Radcliffe-Smith 1986).

Classification is controversial as the Euphorbiaceae is a poorly understood family (Webster 1987). The most recent classification is by Webster (1975). Radcliffe-Smith (1986) has briefly discussed the systems of classification and presents a confusing and complicated situation. Airy Shaw (Willis 1973) states that the classification of the family needs drastic overhauling.

One of the most characteristic features of the family is the fruit which is frequently, although not universally, dehiscent and forms a trilocular regma (Radcliffe-Smith 1986). Also typical of the family are the unisexual flowers which are common to virtually all the genera (Radcliffe-Smith 1986). Many genera within the family produce latex and the occurrence and

types of laticifers have been used in the classification of the family. Rudall (1987) has reviewed the laticifers within the family. Mahlberg et al (1987) have used the triterpenoid content of latex from members of the genus *Euphorbia* for chemotaxonomic purposes.

The family is of importance as a source of food and economic products. Cassava from *Manihot esculente* Crantz is one of the principal food crops in much of Africa, India and Brazil. Castor oil (*Ricinus communis* L.), Tung oil (*Aleurites fordii* Hemsl.) and candlenut oil (*Aleurites molucana* Willd.) are commercial oils from the family. Rubber from *Hevea brasiliensis* is of major importance throughout the world. Rizk (1987) discusses other industrial and commercial products from members of the Euphorbiaceae. Schultes (1987) has discussed the use of species of the family in primitive and advanced societies.

1.1.2 Diterpenes from the Euphorbiaceae.

Diterpenoid compounds have been isolated from many species of the Euphorbiaceae. Of particular interest are the phorbol, and related macrocyclic, diterpenes because of their toxicological and pharmacological properties. The occurrence and properties of these compounds have been reviewed by a number of workers, the most recent being the book "Naturally Occurring Phorbol Esters" edited by Evans (1986). Other reviews include those of Evans and Taylor (1983), Evans and Soper (1978), Hecker (1977), Hecker and Schmidt (1974) and Kinghorn (1979).

The phorbol and related macrocyclic diterpenes have been segregated into two broad groups (Evans 1986): those closely related to phorbol and which have tumour promoting and irritant activity, ie. the tiglane, daphnane and ingenane types (Fig.1.1); and the non-irritant and non-tumour

promoting macrocyclic diterpenes, the casbane, jatrophone, lathyrane, jatropholane, crotofolane and rhamnofolane types (Fig.1.2).

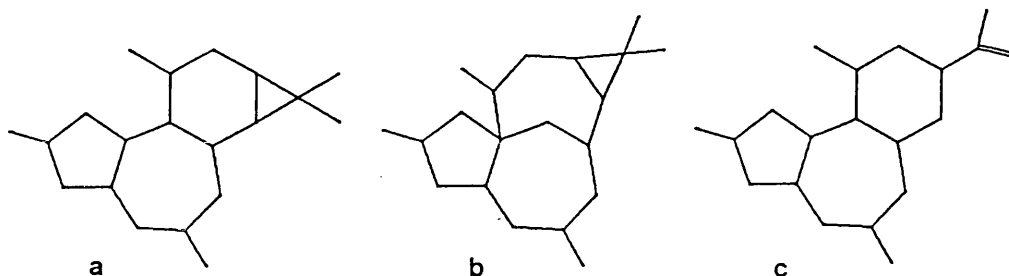


Fig 1.1: The tiglane (a), ingenane (b), and daphnane (c) diterpene skeletons.

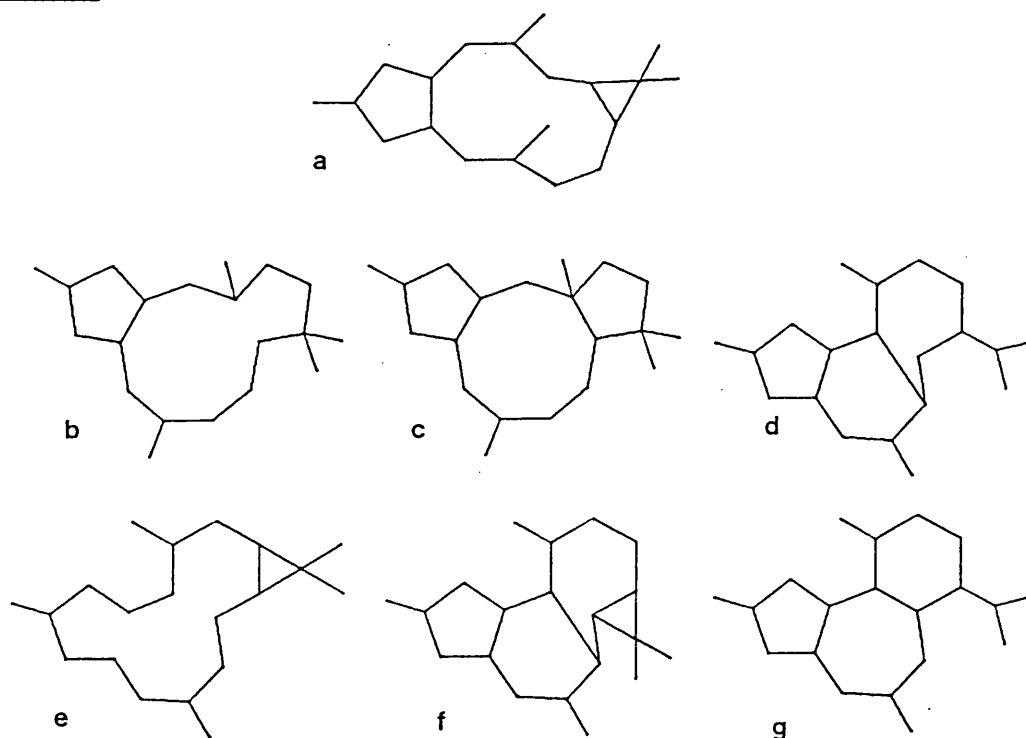


Fig 1.2: The lathyrane (a), jatrophone-a (b), jatrophone-b (c), crotofolane (d), casbane (e), jatropholane (f) and rhamnofolane (g) diterpene skeletons.

Croton oil, from which phorbol was first isolated, was shown to have tumour promoting properties by Berenblum (1941) in his famous two-stage carcinogenesis experiment. He was able to demonstrate that there were factors in croton oil, from *Croton tiglium* L., which were not themselves carcinogenic but were able to promote tumour growth under certain conditions, the most important being regular repetitive application after exposure to a carcinogen. The factors responsible for this effect turned

out to be various esters of the diterpene phorbol. The structure of phorbol was elucidated in 1968 by Hecker (1968), who went on to examine other members of the Euphorbiaceae, especially the genus *Euphorbia*, for similar activity. The plant family Thymelaeaceae was also found to contain related diterpene compounds.

The environmental hazards of exposure to these compounds has been investigated and the role of secondary carcinogenic risk factors in the etiology of environmental cancers discussed by Hecker (1981, Hecker *et al* 1984). It has been shown that honey made from pollen and nectar collected by bees from some *Euphorbia* species contains derivatives of phorbol and ingenol which are capable of tumour promotion (Evans 1986). In China, it has been shown that soil underneath tung oil trees, *Aleurites fordii*, which grow along many of the roads in certain areas contained sufficient activity to activate latent Epstein-Barr virus. E.B.V. has been implicated in some forms of naso-pharyngeal cancer, and there is a correlation between the occurrence of these trees and the incidence of Burkett's Lymphoma in which E.B.V is implicated (Zeng *et al* 1984). Hecker (1984) has shown a correlation between the use of leaves of the bushes *Croton flavens* L. and *Jatropha gossypifolia* L. as a tea and oesophageal cancer on the island of Curacao, near Venezuela. The primary carcinogen in this case was thought to be present in drinking water polluted with oil from a refinery on the island. Use of the herbal teas was considered important in the epidemiology of oesophageal cancer as inhabitants on neighbouring islands, whose water was also polluted but who did not use the same plants for domestic purposes, did not suffer from the same high incidence of cancer of the oesophagus.

St. George's disease in cattle, which occurs in parts of Australia due to

ingestion of certain species of *Pimelia* (Thymelaeaceae), has been shown to be due to a derivative of phorbol, (Roberts *et al* 1975), and is an example of primary toxicity due to the diterpene. Croton oil, once used as a purgative in medical practice, is no longer used because of its toxicity.

Skin irritation and contact dermatitis due to exposure to members of the Euphorbiaceae are well documented (Mitchell and Rook 1979, Watt and Breyer-Brandwijk 1962). The implications in carcinogenesis due to house plants of the Euphorbiaceae are not thought to be significant because of the lack of repeated, regular contact after exposure to a carcinogen.

The pharmacology of the irritant and cocarcinogenic diterpenes has been extensively studied and reviewed (Aitken 1986, Kinsella 1986, Aitken 1987, Kinsella 1987, Blumberg *et al* 1987, Evans and Edwards 1987) and their effects on protein kinase-C elucidated. The macrocyclic diterpenes, however, have not been so extensively examined.

1.1.3 Isolation of diterpenes.

Phorbol was isolated as a hydrolysis product from Croton oil in 1935 and the structure was elucidated in 1968. The first macrocyclic diterpene, a derivative of lathyrol, was isolated from *Euphorbia lathyris* in 1937 and the structure established in 1970 (Zechmeister *et al* 1970).

Separation of individual diterpenes of the Euphorbiaceae requires complex methods and these rely on a combination of partition and chromatographic techniques. Hecker and co-workers routinely use a form of countercurrent distribution, either a Craig or an O'Keefe apparatus (Hecker and Schmidt 1974) which fractionates the crude extract into hydrophobic and hydrophilic portions. They have fractionated crude Croton oil in the

apparatus using a solvent system of petroleum ether-methanol-water and were able to concentrate the biologically active components of the oil into 5% of the starting material. Further fractionation of the hydrophilic material was carried out on a chromatographic column, using deactivated silica and a carbon tetrachloride- ether solvent system, and by countercurrent distribution using a variety of solvent systems.

Evans and co-workers (Evans 1986) rely on a preliminary fractionation involving partitioning a crude extract of plant material against hexane-aqueous methanol, and then further partitioning the aqueous methanol layer against diethyl ether. The less polar triterpenes, sterols and some tri-ester diterpenes partition into the hexane layer, and the more polar diterpenes partition into the ether layer. The net result is similar to that obtained using countercurrent distribution methods, and the resulting fractions are further processed using chromatographic techniques. Evans has used a partition preparative thin layer chromatography technique to purify the compounds. TLC plates made of Kieselguhr were coated with diethylene glycol and developed in a suitable solvent system. The plates were visualised, the appropriate bands scraped off and the compounds separated from the diethylene glycol by extracting with dichloromethane and partitioning against dilute NaCl solution (Evans and Taylor 1983).

Kinghorn and co-workers (Lin *et al* 1983a, b, c) have used droplet countercurrent chromatography as a partition technique for the fractionation of crude extracts of diterpenes. Evans and Taylor (1983) however, regard the technique as being less successful than their method of partition preparative thin layer chromatography.

Bauer *et al* (1983) have developed an HPLC system utilising a bonded phase C18 column for screening plants for the presence of diterpene esters. Borris *et al* (1984) used an initial partitioning procedure followed by chromatography on silica gel and sephadex LH-20, to yield a series of daphnane ortho-esters from the roots of *Gnidia kraussiana* L. (Thymelaeaceae). Connolly *et al* (1984a) used the method of Evans to produce an ether soluble extract of *Euphorbia kamerunica*, and isolated five ingol esters by multiple preparative TLC. Roberts *et al* (1975) isolated a daphnane ortho-ester from *Pimelea simplex* (Thymelaeaceae) by extracting dried plant material with 95% ethanol, followed by repeated column chromatography on Sephadex LH-20 and preparative thin layer chromatography.

1.2 Nuclear magnetic resonance spectroscopy in structure elucidation.

1.2.1 Background.

Nuclear magnetic resonance (NMR) was first detected in 1945 by two independent groups of workers led by Bloch at Stanford University and Purcell at Harvard. The first commercial proton NMR spectrometer was introduced in 1953 and the development of magnet and computer technology has led to the very powerful technique that we know today.

Fourier transform (FT) NMR became possible with the advent of modern computer technology. Using FT techniques the NMR properties of low abundance magnetic isotopes, such as ^{13}C , can be exploited to produce meaningful spectra. Multiple scan accumulations to improve the signal to noise ratio, impractical because of time limitations in continuous wave instruments, have become routine. It has become possible to obtain high resolution spectra for extremely small sample sizes. In proton NMR spectroscopy, spectra for submilligram quantities of small molecules (less than MW=1000) and in ^{13}C , milligram quantities, can be obtained. FT and computer technology have aided the development of two dimensional NMR spectroscopy, probably the most important development in structure elucidation in the last fifteen years.

The development of magnet technology, particularly superconducting magnets, which provide the very stable, high magnetic fields necessary for NMR, have allowed the development of very high resolution spectrometers with operating frequencies for ^1H of up to 600MHz.

The ability of a nucleus to undergo magnetic resonance depends

fundamentally on its nuclear spin (I). The two nuclei which are of prime concern in the structure elucidation of organic plant constituents are ^1H and ^{13}C , both of which have nuclear spin values of $1/2$. When placed in a magnetic field, such nuclei will be confined by quantum mechanical considerations to two energy levels only. Irradiation with radiofrequency results in transitions between these two energy levels.

When a compound is placed in a magnetic field and irradiated with a short pulse of radiofrequency energy which excites all the nuclei simultaneously, the nuclei re-emit the radiation at a frequency particular to each nucleus. The free induction decay is the combined radiofrequency emissions from the nuclei which is collected and digitised by the spectrometer's computer. Fourier transformation of the time domain FID results in the conventional frequency domain spectrum.

The electron density about each nucleus will influence the radiofrequency at which that nucleus will resonate. The electrons surrounding the nucleus shield it from the radiofrequency, and the net field experienced by the nucleus will depend on the degree of shielding exerted by the electrons. Nuclei in environments of different electron density will thus resonate at different radiofrequencies. The nuclear shielding is proportional to the applied field. The chemical shift for a particular nucleus is defined as the nuclear shielding divided by the applied field and is measured relative to a reference nucleus, commonly tetramethylsilane in organic solvents and DSS (sodium 2,2-dimethyl-2-silapentane-5-sulphonate) in aqueous solvents. Nuclei in different environments will therefore be shifted up- or downfield of the reference compound. The spectrum is conventionally scaled in the units of chemical shift (parts per million) from the reference standard.

Further information in the spectrum is provided by spin-spin coupling (splitting) patterns caused by the interaction between neighbouring magnetic nuclei. In proton magnetic resonance the major splitting patterns are due to protons attached to adjacent carbon atoms, i.e. three bond vicinal couplings and, if the protons on a particular carbon atom are non-equivalent, two bond geminal couplings. Longer range couplings over four or five bonds can be observed in some cases.

^{13}C NMR spectra are conventionally decoupled from proton-carbon spin-spin coupling effects by double irradiation techniques for simplification. Special pulse sequences are available which alter the phase of signals depending on multiplicity.

A number of books and reviews on the theory and application of NMR techniques are available, of which the following are of particular interest: Shoolery (1984), Kemp (1986), Derome (1987), Morris (1987), Sanders and Hunter (1987) and Branch and Casy (1988).

1.2.2 Applications of one dimensional NMR spectroscopy.

The one dimensional proton spectrum is the quickest and simplest to obtain. Many laboratories are equipped with low resolution, 60MHz continuous wave instruments for fast preliminary screening of samples prior to more detailed investigation. In the isolation procedure, if the compounds of interest have characteristic NMR features, then this form of spectroscopy can be of considerable use in selecting potentially interesting fractions for further purification. Diterpenoid compounds are fairly readily distinguished from triterpenes or sterols commonly found in plant extracts by the presence of deshielded signals downfield of the

aliphatic region between 0.5ppm and 2ppm, and the relatively few signals in the aliphatic region. In this work, the compounds of interest were diterpenoid esters of the phorbol and related macrocyclic types which, in addition to the above example, commonly have acetate functional groups associated with them which are readily detected even on low resolution instruments. In phorbol itself (Fig 1.3), the olefinic proton at C1 is highly deshielded and gives a broad singlet at 7.54ppm (Evans 1986). This signal has been suggested as a reasonable indication of the presence of phorbol and its analogues possessing an alpha-beta unsaturated carbonyl in ring A (Pieters and Vlietinck 1986).

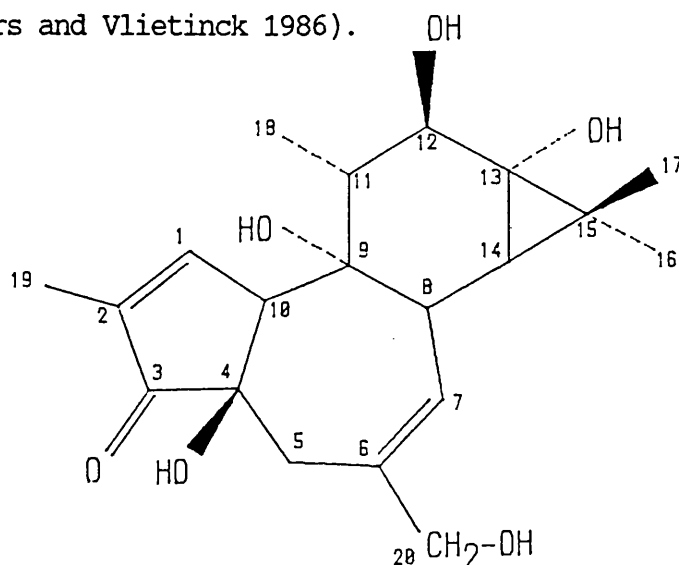


Fig 1.3: Phorbol.

The high resolution ^1H NMR spectrum is usually the first to be obtained for a newly isolated compound. Analysis of the chemical shifts, multiplicities and integrals for the peaks gives an idea of the type of compound, the possible number of methyl groups, any acetate substituents that may be present and the degree of unsaturation and presence of aromaticity. Measurement of coupling constants for various peaks may give an indication of which peaks are coupled and decoupling experiments will serve to confirm this as well as to reveal hidden detail under the decoupled area.

^{13}C spectra take longer to accumulate, and it is common to perform a series of three experiments to ascertain the degree of proton substitution on each nucleus. The 90 and 135 DEPT (Distortionless Enhancement by Polarisation Transfer) multipulse experiments alter the phase relationships between CH_3 , CH_2 , and CH ^{13}C signals and allow sub-spectra to be produced which differentiate between the three types of nucleus. The 135 DEPT experiment produces a plot having the CH and CH_3 carbon atoms signals phased normally and pointing upwards, and CH_2 signals pointing downwards. The 90 DEPT experiment eliminates CH_2 and CH_3 signals entirely, leaving only CH signals on the plot. The resulting spectra can be correlated and provide extremely useful information on the gross structure of the compound under investigation.

1.2.3 Applications of two-dimensional NMR spectroscopy.

Complex one-dimensional spectra in which peaks are not fully resolved or where coupling constants are of similar values can be difficult to assign even with spin-spin decoupling, which can be time consuming. The presentation of such information in a second dimension would be extremely useful.

The first 2D NMR experiment was proposed by Jeener in 1971 and was a multipulse experiment in which the nucleus under examination was excited with a primary pulse, followed by a time interval, t_1 , after which a second pulse was applied followed by a second time interval, t_2 , during which the FID was collected. The value for t_1 is incremented over a range of time and a data matrix collated.

The signal intensity in t_2 after Fourier transformation for a particular nucleus is influenced by the length of the time interval, t_1 , and the

signal intensity in t_2 varies sinusoidally with t_1 . Coupled nuclei emit frequencies in phase with each other, so that when a series of plots, one for each time interval, t_1 , is produced, those peaks which are coupled to each other will be present on the same line plot, whereas those which are not coupled are absent. The experiment produces a data matrix of one time domain, t_1 , against the other, t_2 , which, after a second Fourier transformation, produces a two dimensional plot of frequency vs frequency, i.e. chemical shift vs chemical shift.

Such plots are routinely presented as contour plots which give the equivalent of the one dimensional spectrum along the diagonal with coupled nuclei showing as off-diagonal peaks. Analysis of such plots is generally far simpler than in one dimensional spectra, and the connectivities between coupled peaks can be traced out on the plot.

Double Quantum Filtration (DQF) is a technique which utilises the phenomenon of multiple quantum coherence, in which a strong level of irradiation excites a double quantum transition, which can only be generated in coupled systems. In DQF COSY a plot is produced in which singlets are eliminated thus simplifying the correlation. Non coupled peaks include the strong singlets due to acetate and tertiary methyl groups.

There are two basic types of two dimensional NMR spectroscopy - correlation spectroscopy and J -resolved spectroscopy. Correlation spectroscopy is the more useful of the two in structure elucidation with proton-proton COSY the most frequently used. J -resolved 2D experiments are more restricted in their applications (Derome 1987).

^{13}C - ^{13}C COSY would be extremely useful for working out the basic carbon skeleton of a compound. However, due to the low natural abundance of the ^{13}C isotope (approx. 1%) the direct analogy to ^1H - ^1H COSY is not possible. However, another two dimensional technique, 2D INADEQUATE (Incredible Natural Abundance Double Quantum Transfer Experiment), although insensitive, is feasible. The problem lies in the fact that the chances of having two ^{13}C nuclei adjacent to each other is about 1:10 000 and this means that large sample sizes are required. The technique utilises multiple quantum coherence effects and produces a plot which reflects connectivities due to couplings between adjacent carbon atoms in the molecule.

Dekker *et al*(1987) have used the technique to elucidate the structure of an unusual daphnane derivative from *Jatropha zeyheri* (Euphorbiaceae). The experimental conditions were extreme: a 58 hour accumulation time, a large matrix (512x4096 data points), a very high resolution instrument (100MHz) and a 150mg in 0.3ml sample to which chromium tris-acetylacetonate was added to reduce the relaxation times of the nuclei. The results were impressive- all the C-C connectivities could be traced and the structure established.

Heteronuclear shift correlations, such as C-H Shift correlation, are useful in determining the connectivities between the protons and carbon atoms in a molecule. In conjunction with the information from the 90 and 135 DEPT experiments, it can provide useful information on gross structure. Long range C-H Shift correlations using coupling constants for four or five bond couplings can be useful in determining the points of substitution of functional groups. Connolly *et al* (1984c) have used long range C-H couplings to assign the ester linkages on a new 19-hydroxyingol

from *Euphorbia poissonii*.

J-resolved two dimensional spectroscopy is useful in clarifying the coupling constants in spectra where there is a strong overlap of peaks and can be used in conjunction with the information obtained from other NMR experiments.

1.2.4 Complementary techniques in structure elucidation.

Phorbol esters are not amenable to elemental analysis because the compounds solvate strongly (Evans 1986) and thus alternative methods for accurate mass determinations are sought. Mass spectrometry (MS) can be useful for this purpose if a suitable molecular ion is produced. The technique is of great importance in determining the nature and degree of acyl substitution on diterpene esters as the acyl groups tend to fragment in a predictable manner which can be analysed. In a successful MS experiment, the molecular ion for the entire molecule, and thus the empirical formula, and the ion for the de-esterified parent alcohol can be obtained, yielding valuable information on the structure of the compound.

In the structure elucidation of diterpenoid esters such as those commonly found in the Euphorbiaceae, mass spectrometry in conjunction with NMR forms the basis of structure elucidation. Crystalline material may be subjected to X-ray crystallographic techniques which will confirm both the gross structure and the solid state conformation of the molecule. Such an investigation is the exception rather than the rule as the technique is a specialist one and not always available to the phytochemist.

Other spectroscopic techniques, such as infra-red and ultra-violet spectroscopy are of limited value in the structure elucidation of

diterpenoids. They are extremely useful in confirming the presence or absence of particular functional groups but do not reveal a significant amount of overall detail at the structural level.

CHAPTER TWO.

INVESTIGATION OF *SYNADENIUM COMPACTUM*.

2.1 Occurrence of diterpene esters within the Euphorbioideae.

Webster (1975) presents the following structure of the family Euphorbiaceae, divided into five sub-families and fifty two tribes:

Sub-families:

1. Phyllanthoideae
2. Oldfieldioideae
3. Acalyphoideae
4. Crotonoideae
5. Euphorbioideae

Synadenium compactum occurs within the subfamily Euphorbioideae. Tribes and genera within this subfamily are classified as follows (genera are grouped into sub-tribes):

Tribe 48. Stomatocalyceae.

Stomatocalyx, *Plagiostylus*, *Hamilcoa*, *Nealchornia*.

Tribe 49. Hippomaneae.

Mabea, *Senefeldera*, *Senefelderopsis*.

Carumbium.

Hippomane, *Actinostemon*, *Adenopeltis*, *Bonania*, *Colliguaja*, *Dalembertia*,
Duvigneaudia, *Excoecaria*, *Glyphostylis*, *Grimmeodendron*, *Gymnanthes*,
Maprounea, *Sapium*, *Sebastiania*, *Spirostachys*, *Stillingia*.

Tribe 50. Pachystromateae.

Pachystroma.

Tribe 51. Hureae.

Hura, *Algernonia*, *Ophthalmoblapton*, *Tetraplandra*.

Tribe 52. Euphorbieae.

Anthostema, *Dichostemma*.

Neoguillauminia, *Calycopeplus*.

Euphorbia, *Chamaesyce*, *Cubanthus*, *Endadenium*, *Monadenium*, *Pedilanthus*, *Synadenium*.

Kinghorn (1979) has documented the distribution of irritant genera within the family Euphorbiaceae on the basis of either chemical investigation or reports of use as purgative, arrow or fish poison, irritant or wart cure. Within the subfamily Euphorbioideae he includes the following genera: *Hippomane*, *Excoecaria*, *Grimmeodendron*, *Sapium*, *Sebastiania*, *Stillingia*, *Hura*, *Anthostema*, *Euphorbia*, *Pedilanthus* and *Synadenium*.

The genus *Euphorbia* has been extensively studied and is the primary source of ingenane type diterpenes. The distribution of diterpenes within the genus has been discussed in the reviews on the family (Hecker 1977, Evans and Soper 1978, Evans and Taylor 1983, Evans 1986). Evans and Kinghorn (1977) have compared the occurrence of diterpenes within the genera *Euphorbia* and *Elaeophorbia* and have proposed that *Elaeophorbia* be included

in the genus *Euphorbia*.

Adolf and Hecker (1984) have identified a number of 13,20-diesters of 12-deoxyphorbol as well as orthoester derivatives of resiniferonol, a daphnane diterpenoid having an isopropenyl group at carbon 13 instead of a cyclopropane ring, from *Hippomane mancinella* L., a highly toxic tree native to central America and the West Indies. Taylor *et al* (1981) have isolated a number of 4-deoxy-5-hydroxyphorbol and 4,20-dideoxy-5-hydroxyphorbol derivatives having an unusual nitrogen containing ester group at position 12 on the skeleton, from *Sapium indicum*. Seip *et al* (1983) have isolated derivatives of 12-deoxyphorbol, phorbol and 6 β ,7 β -epoxyphorbol from *Sapium sebiferum* Roxb. Three *Excoecaria* species from Thailand have been shown to possess resiniferonol orthoester derivatives, one of which is the same as that from *Hippomane mancinella* (Wiriyachitra *et al* 1985). Kinghorn (1980) has shown the presence of a skin irritant 4-deoxyphorbol derivative from *Synadenium grantii* Hook f. Adolf and Hecker (1980) have demonstrated the presence of daphnane and tiglliane orthoesters in *Stillingia sylvatica* L. Similar orthoester diterpenes have been isolated from *Hura crepitans* (Sakata *et al* 1971). The presence of phorbol derivatives has been detected in a species of *Mabea* which was responsible for contact dermatitis amongst soldiers serving in Belize (Schmidt 1987).

In addition, using the same criteria as Kinghorn for toxicity, *Maprounea*, *Monadenium*, *Spirostachys* (Watt and Breyer-Brandwijk 1962), *Pedilanthus* and *Chamaesyce* (Hartwell 1969) are likely to contain phorbol or related diterpenes. Latex of *Pedilanthus tithymaloides* Poit. has been reported to cause conjunctivitis but not dermatitis (Lim and Soepadmo 1984). Fifteen out of forty genera within the subfamily have either been shown to contain

toxic diterpenes or are likely to contain them on the evidence of ethnobotanical or other reports.

The type genus for the family, *Euphorbia*, has been the most extensively studied of all the genera within the Euphorbiaceae. Most detailed biochemical investigation has centred on the properties of the irritant and cocarcinogenic diterpenoids with relatively little work being done on the macrocyclic, non-irritant compounds. Many of these compounds have been tested with *in vivo* systems and a large number found to be cytotoxic. Abo (1982) has tested a variety of ingol derivatives, both naturally occurring and semi-synthetic, and has shown that diesters of ingol exhibit greater cytotoxicity than tri- or tetraesters. Evans (1986) has suggested that it is the macrocyclic compounds which are responsible for the anti-cancer activity of some members of the Euphorbiaceae, and not the irritant, cocarcinogenic diterpenoids.

Synadenium Boiss. is a genus comprising 15 species of succulent, branched small trees or shrubs occurring from eastern to southern Africa (Willis 1973). Many of the species of *Synadenium* have been used in ethnopharmacological practice (Watt and Breyer-Brandwijk 1962). The typical uses as fish poison, purgative and vesicant suggest the presence of irritant and cocarcinogenic diterpenes in some of the species. *Synadenium grantii* Hook f. is the best known of the *Synadenium* species, and is grown as a house plant in Europe and north America (Kinghorn 1980). It was first reported as an irritant species by Rook (1965). A case report on the ingestion of fresh material by a child has been presented (Spoerke *et al* 1985). Kinghorn (1980) has isolated a toxic phorbol derivative, 12-*O*-tigloyl-4-deoxyphorbol -13-isobutyrate (Fig.2.1) from the species.

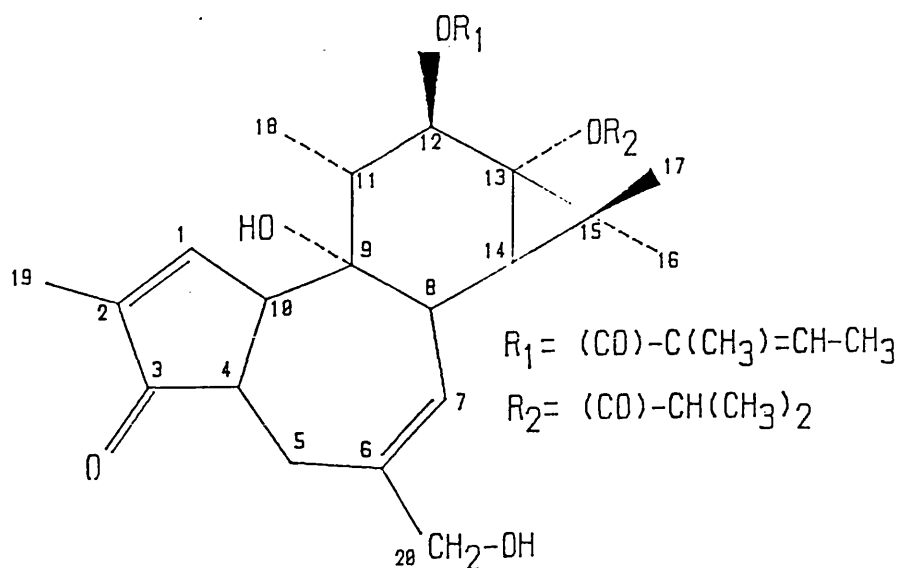


Fig. 2.1: 12-O-tigloyl-4-deoxyphorbol-13-isobutyrate, isolated from *Synadenium grantii* (Kinghorn 1980).

Of the other species of *Synadenium*, *S. cupulare* L.C. Wheeler is the most commonly referred to. The toxicity of the plant is reflected in its common names, Sheba valley death tree (Jacobsen 1977) and dead man's tree. In Zulu tradition the tree is said to lure people and animals to it in order to kill them ! (Palmer *et al* 1972). Kokwaro (1976) has recorded the use of *S. compactum* in the treatment of calves, but does not give details of either the condition being treated or the methods of preparing the plant material.

Synadenium compactum is a many branched small tree or shrub native to Kenya and East Africa. The leaves are broadly obovate, glabrous, minutely toothed with a sharply keeled midrib. The entire inflorescence is red and densely pubescent. The styles are short, bifid and thickened (Holmes 1987). The plant has been introduced to other parts of the world as a garden shrub on account of its ornamental foliage. *S. compactum* var. *rubrum* is the most commonly grown variety by virtue of its purplish red leaves. The plant from which material was collected for this work is *S. compactum* N.E.Br. var. *compactum* which has dark green leaves with flaking, almost silver coloured bark.

2.2 Materials and Methods.

2.2.1 Materials.

Latex from *Synadenium compactum* was collected near Manzini, Swaziland in December 1984, December 1985 and March 1987. The material was collected into methanol and stored below 0° until use. The species was identified by Mrs S. Holmes of the Royal Botanic Gardens, Kew, Richmond, Surrey, and voucher specimens and photographs of the tree in flower have been placed in the herbarium at Kew.

All solvents used were of standard laboratory grade and were redistilled in glass before use.

2.2.2 Extraction.

Latex (approx. 250ml) of *Synadenium compactum* was collected into methanol. The latex/methanol (50:50) mixture was evaporated under reduced pressure to a soft mass. This was extracted with acetone (6x300ml) and filtered. The solid residue was discarded. The acetone extract was evaporated to dryness under reduced pressure at 40°.

2.2.3 Partitioning.

The resinous acetone extract was redissolved in methanol-water 90:10 and partitioned against *n*-hexane (3x200ml). The hexane fractions were combined and backwashed with a further two portions of methanol-water 90:10 (100ml) and evaporated to a small volume under reduced pressure at 40°. The aqueous methanol fractions were combined.

Equal portions of diethyl ether and water (500ml) were added to the aqueous methanol fraction. The ether layer was separated and the aqueous

layer partitioned against a further four portions of fresh diethyl ether (300ml). The ether fractions were bulked and extracted with two portions of a 1% aqueous sodium carbonate solution (100ml). The ether fraction was dried over anhydrous magnesium sulphate and evaporated to dryness under reduced pressure at 40°. The aqueous fractions were discarded.

2.2.4 Fractionation techniques and monitoring of fractions.

2.2.4.1 Column chromatography.

Standard gravity column, liquid chromatography techniques were used in which the adsorbent, either silica gel or florisil, was slurried in the eluting solvent, and poured into glass chromatography columns. Fractions were collected by volume, and monitored by thin layer chromatography (0.2mm silica gel plates, E. Merck, supplied by B.D.H., Poole, Dorset.) in one of the following solvent systems:

- i. Hexane: ethyl acetate: diethyl ether 1:1:1
- ii. Toluene: ethyl acetate: diethyl ether 10:2:2
- iii. Toluene: ethyl acetate: diethyl ether 1:1:1
- iv. Chloroform: ethyl acetate 90:10
- v. Toluene: diethyl ether 70:30
- vi. Toluene: ethyl acetate: diethyl ether: methanol 50:20:20:10
- vii. Toluene: ethyl acetate: diethyl ether 100:25:25
- viii. Chloroform:methanol 95:5
- ix. Hexane: diethyl ether 20:80
- x. Toluene: diethyl ether 1:1
- xi. Hexane: chloroform: methanol 50:95:5

2.2.4.2 Droplet countercurrent chromatography.

Droplet countercurrent chromatography was carried out on a Buchi

B-670 instrument (Orme Scientific, Manchester.) equipped with 2.7mm i.d. glass columns. The methods and operation of the technique are described in detail in Part 2 of this thesis.

2.2.4.3 Flash column chromatography.

The glass flash chromatography column, (Aldrich Chemical Company, Gillingham, U.K.) was dry filled with silica gel G 230-400 mesh (40g) and a 1mm layer of sand (chromatography grade) placed at the top of the packing. The sample was applied to the dry bed and the solvent introduced. The reservoir was filled with solvent and pressure applied from a nitrogen cylinder. The nitrogen pressure was controlled using a needle valve in line from the cylinder. Fractions were collected by volume using a 5ml glass syphon receiver. Hostettmann *et al*(1986) have discussed the applications and principles of flash column chromatography.

The fractions from the flash columns were analysed by TLC using the same solvent systems as for column chromatography.

2.2.4.4 Preparative High Performance Liquid Chromatography.

Preparative high performance liquid chromatography was carried out on a Dupont Model 830 Preparative HPLC system (Dupont Instruments, Stevenage.) using standard techniques with a 25x2.1 cm silica gel column (Zorbax-SIL particle size 8-15 micrometers). A 1ml injection loop was used with approximately 25mg of sample being injected each time. The flow rate was set at 20ml/min and the eluent passed through a ultra violet spectrophotometric detector (UV) set at 225nm. Fractions were collected by visual monitoring of the UV trace and manual collection into a round bottomed flask. The solvent was removed under reduced pressure.

2.2.4.5 Centrifugal thin layer chromatography.

Centrifugal thin layer chromatography (cTLC) was carried out on a Harrison Research Model 7924T Chromatotron (T.C. Research, Norwich.), which has an inclined rotor and operates under a positive pressure of nitrogen. Two types of Chromatotron plate were prepared: plain silica gel and silica gel slurried in polyethylene glycol 6000. The plates were prepared as follows:

The rotor was prepared by washing in soap solution, drying and applying a band of masking tape around the edge of the disc to hold the silica slurry. Silica gel (65g) was slurried in distilled water (130ml chilled to 5°), and poured onto the glass rotor. After pouring, the plate was tapped to remove air bubbles and allowed to stand overnight under a plastic cover to ensure even drying of the plate. The dry plate was scraped using the disc scraping apparatus provided to a final thickness of 2mm. The procedure for preparing the polyethylene glycol plates was the same as for the silica plates except that the silica gel was slurried in a solution of PEG6000 (9g in 130ml water).

The sample was applied to the centre of the dry chromatotron plate followed by solvent. The flow rate was adjusted to 6ml/min by raising or lowering the solvent reservoir as required. Samples were collected by time and were tested by TLC in the same solvent systems as for column chromatography.

2.2.4.6 Gas-liquid chromatography.

Fractions from the various preparative procedures were monitored by TLC and/or gas-liquid chromatography (GLC). Two instruments were used, one, a Perkin Elmer F-33 instrument, (Perkin Elmer Ltd, Beaconsfield, Bucks.) having a packed 2m SE-30 column and the other, a Perkin Elmer

Sigma-3 instrument, having a 39m x 0.25mm OV-1 capillary column. Operating conditions for two columns were the same with the oven temperature set at 280° and the injector/detector temperature set at 300°.

2.2.5 Spectroscopic methods.

The NMR instruments used were a Jeol GX400 and GX270 F.T. spectrometers (Jeol UK Ltd, London), or a Jeol PMX60SI C.W. spectrometer. All spectra were obtained in CDCl₃.

The 399.65MHz proton spectra for Sy215/1 and Sy134/6 were collected with 128 scans using a frequency width of 4000Hz and 32K data points to give a digital resolution of 0.24Hz/pt. The 270.05MHz spectra for Sy204/3 and Sy134/4 were obtained with 64 scans for Sy204/3 and 256 scans for Sy134/4 over a frequency width of 3001.2 Hz and 32K data points to give a resolution of 0.18Hz/pt. The 67.8MHz noise-decoupled ¹³C and DEPT spectra were collected with a frequency width of 18050.5Hz and 16K data points to give a resolution of 2.2Hz/pt. 26502, 3600, 43205, 2800 and 18370 scans were accumulated for the ¹³C spectra of Sy215/1, Sy134/6, Sy204/3, Sy134/4 and Sy124/2 respectively.

Two dimensional spectra were obtained using standard pulse sequences (Morris 1986) available on the instruments. The 399.65MHz COSY spectra were zero filled once in the column direction to give a final data matrix of 1024x512 complex points. A Blackman-Harris window function was applied before transformation. The number of scans per slice, frequency width (Hz) and corresponding resolution (Hz/pt) for the four compounds studied were as follows: Sy215/1 (128, 3201.0, 6.2); Sy134/6 (16, 2100.8, 4.1); Sy204/3 (16, 2100.8, 4.1) and Sy134/4 (96, 2200, 4.3).

The 399.65MHz long-range COSY spectrum for Sy215/1 was run under the same conditions as the COSY experiment above but with a fixed delay inserted in the pulse sequence to optimise small couplings. 64 scans were accumulated with a frequency width of 2100.8Hz to give a resolution of 4.1Hz.

The 67.8MHz ^{13}C - ^1H shift correlation spectra were zero-filled once in the column direction to give final matrices of 1024x128 complex points for Sy215/1 and Sy134/6. For Sy134/6, 48 scans were accumulated for each slice with row and column frequencies of 13404.8 and 1600.0Hz giving resolutions of 26.2Hz/pt and 12.5Hz/pt respectively.

Mass Spectrometric measurements were made on a V.G.7070E instrument (V.G. Analytical Ltd., Manchester.) housed in the School of Chemistry at the University of Bath.

2.3 Fractionation of latex.

Three batches of latex were collected on different occasions. Each batch was extracted and partitioned separately using different combinations of techniques and the procedure for each is discussed with its results.

2.3.1 Batch 1 of latex.

250ml of fresh latex was collected into methanol, and stored at 0° before use. The methanol was evaporated under reduced pressure and the remaining soft mass subjected to the partitioning procedure described above to provide a hexane soluble extract (18g) and an ether soluble extract (6g). The remaining water soluble extract (0.3g) was discarded.

The ether soluble extract (6g) was divided into four equal portions, and each portion was further partitioned on a Florisil (200-300 mesh) gravity column (120g), eluted with a step gradient of *n*-hexane-ethyl acetate, and the eluate collected in 10ml fractions.

Fractions were examined by thin layer chromatography in the solvent system cyclohexane:ethyl acetate:diethyl ether (1:1:1) and visualised by spraying with 60% sulphuric acid and heating at 110° for 5 min. Like fractions from the first three columns were combined and given the code Sy26 which comprised 16 fractions. The fractions could be categorised into three main types from TLC. Those with R_f values between 0.57 and 0.49 coloured red and turned purple on standing. They are likely to be mixtures of sterols and triterpenes. Those with R_f values between 0.49 and 0.35 coloured green and seemed to be the major group, whereas those between R_f values of 0.35 and 0.30 coloured yellow and orange and appeared to be minor components.

The fractions from the fourth column were coded Sy31 and were dissolved in *n*-hexane. Samples that were not used at once were stored in a freezer below 0°. After 24 months, the unused fractions were examined. In one vial, corresponding to fractions Sy31/47 to Sy31/56, colourless prisms had crystallised out. The material was filtered off and weighed. The crystals were coded Sy215/1 (12.9mg). A 2mg portion was removed for NMR, one crystal for MS and the balance retained for X-ray crystallography.

The fractions from the combined columns were redissolved in cyclohexane. Fractions Sy26/9 to Sy26/12 gave an immediate precipitate. This was filtered off and combined with like fractions from the fourth column Sy31/37 to Sy31/40. The combined material (200mg) was re-chromatographed on Florisil (30g) using the same conditions as for the first fractionation. The resulting fractions were given the code Sy52. Fractions Sy52/8 (6mg), Sy52/9 (35mg) and Sy52/10 (35mg) were combined and redissolved in cyclohexane. The resulting precipitate was filtered off and the insoluble material further recrystallised from cyclohexane. The fractions from the recrystallisation were coded Sy65/1, 2 and 3 and were tested by GLC using a SE-30 column at 280°. The fraction Sy65/2 (16.6mg) gave a primary peak with a retention of 5.8min which appeared to be one compound, with a few minor impurities. MS and NMR experiments were carried out on this material.

The mother-liquors from Sy26/9 to Sy26/12 were recombined (300mg) and re-chromatographed on Florisil (30g), using a gradient elution of cyclohexane and ethyl acetate. Seventeen fractions, coded Sy54, were collected. One of these, Sy54/8 (7mg), appeared to be a single compound when tested by TLC. It was dried under vacuum and submitted for MS and

NMR. The remaining fractions were not well separated, and fractions Sy54/10 to Sy54/16 were recombined and an attempt was made to separate them using droplet countercurrent chromatography. This was unsuccessful and the fractions were recombined.

Fractions Sy26/4 to Sy26/8 were combined with Sy31/23 to Sy31/36 and redissolved in cyclohexane. The precipitate was washed with portions of cold cyclohexane and the resulting insoluble material, Sy63/1 (10mg), tested by GLC. The major peak had a retention of 4.1min on a SE-30 column and a number of minor impurities were apparent. NMR and MS experiments were run on the material.

2.3.2 Batch 2 of latex.

250ml of fresh latex was collected into methanol, and stored below 0° before use. The methanol was evaporated under reduced pressure and the remaining soft mass subjected to the partitioning procedure described above to provide a hexane soluble extract (21g) and an ether soluble extract (4g). The water soluble extract was discarded.

The entire ether soluble fraction was redissolved in *n*-hexane and placed in a refrigerator (4°) overnight. The precipitate (156mg) was filtered off.

The mother-liquor was evaporated to dryness, and placed on a Florisil 60-100 mesh (100g) column and eluted with a solvent gradient of *n*-hexane and ethyl acetate. The fractions from the column (Sy102) were combined according to the TLC profile, and were redissolved in *n*-hexane. Precipitates were recorded in fractions Sy102/16 to Sy102/21.

The precipitate from the ether soluble fraction was combined with fractions Sy102/16 to Sy102/21 (total weight 674mg) and placed on a Florisil 100-200 mesh (50g) column and eluted with a solvent gradient of toluene and ethyl acetate. Eighteen fractions (Sy105) were collected. Two of the fractions from this column, Sy105/8 and Sy105/9, were combined (488mg) and separated by droplet countercurrent chromatography. The lower phase of the solvent system *n*-hexane:toluene:diethyl ether:methanol:water (10:4:4:10:1) was designated the mobile phase. The apparatus was fitted with 488 tubes and set at a flow rate of 7ml per hour. Fractions were collected at 60min intervals and were evaporated to a small volume under reduced pressure. Ethyl acetate was added and the solution dried over anhydrous magnesium sulphate. After filtering, the fraction was evaporated

to dryness under reduced pressure. 159 fractions were collected tested by TLC and combined where necessary. One fraction, Sy106/131-150 (21mg) appeared to be pure by TLC. It was recoded Sy107/2 for convenience and submitted for NMR and MS.

Fractions Sy106/95 to Sy106/113 were recombined and fractionated using flash column chromatography. Silica Gel 230-400mesh (38g) was dry-loaded into a glass flash chromatography column. The sample (240mg) was applied to the top of the dry column which was then eluted with chloroform:ethyl acetate 90:10 (100ml) and chloroform:ethyl acetate 80:20 (100ml). The flow rate was adjusted to 5ml per min and fractions were collected at 1min intervals. 32 fractions were collected. Fractions 12 to 14 appeared pure by TLC and were recombined. The sample (16mg) was given the code Sy124/2 and was submitted for NMR and MS.

All the remaining fractions from both batches of latex which showed green coloured compounds by TLC between R_f 0.57 and 0.35 in the solvent system hexane:ethyl acetate:diethyl ether (1:1:1) were recombined and subjected to preparative HPLC.

Analytical HPLC (Zorbax-SIL column 10cmx0.5cm) on samples of the fractions had demonstrated that the compounds could be separated more easily than on gravity columns or by other methods. Accordingly, the system was scaled up to use a 25cmx21.2mm preparative column. The combined fractions (480mg) were prepared by dissolving them in 20ml of *n*-hexane:tetrahydrofuran (1:1). A 1ml injection loop was used and a flow rate of 20ml/min. The eluate was monitored with a UV detector set at 225nm. The chart recorder was set at 5min/cm.

Fractions were collected from the column by visual monitoring of the chart recorder. Seven fractions were collected and were analysed by capillary GLC using a 39m OV-1 column (0.25mm i.d.), at an oven temperature of 280°. The profile of the mixture before separation is shown in Fig 2.2. There are seven major signals and a further seven or eight minor ones. The major compound has a retention time of 9.8min and the second major compound a retention time of 6.4min.

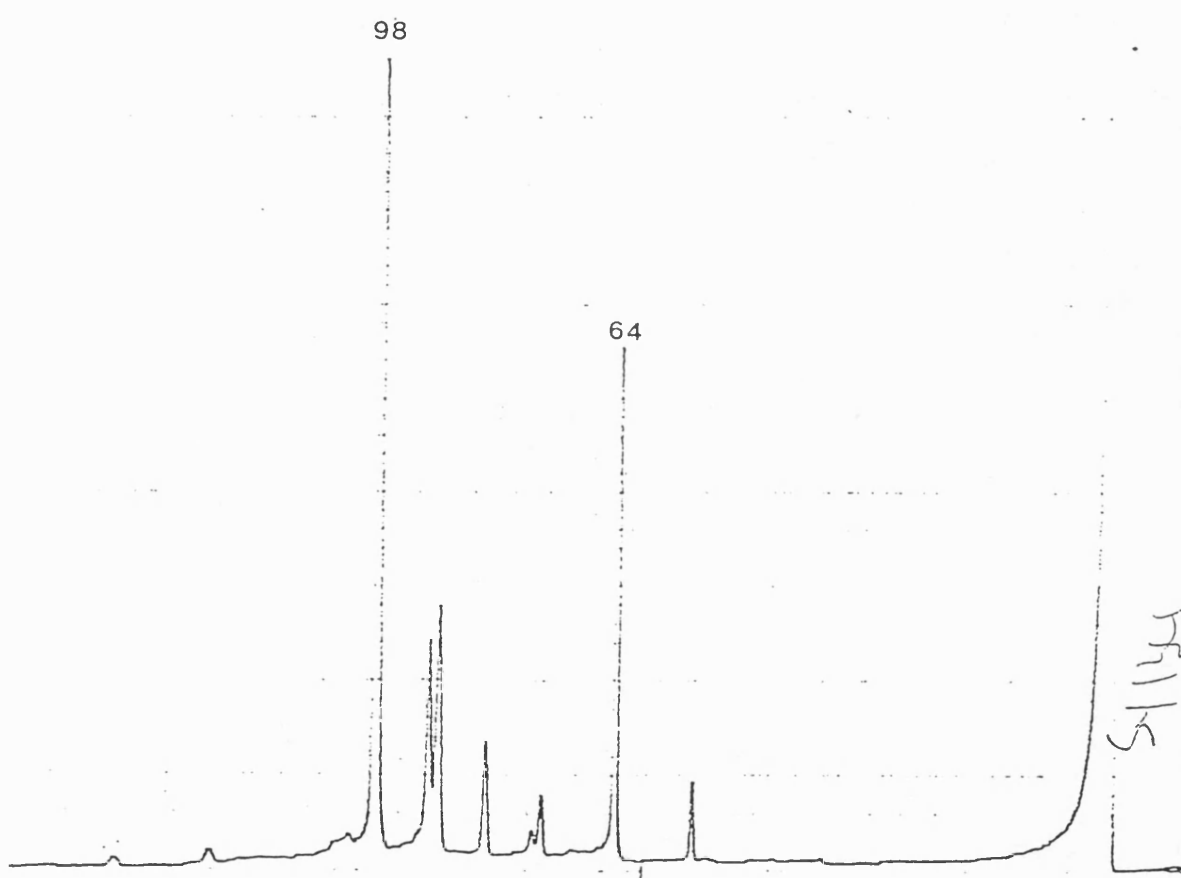


Fig 2.2: GLC profile of fraction Sy134 before separation by preparative HPLC. The two major peaks are marked, 64mm and 98mm after the solvent peak.

Of the fractions separated on the column, only two were judged pure enough for NMR and MS. The trace of the HPLC preparative separation is shown in Fig 2.3 and is marked to show the collection slices for each fraction. Sy134/6 (65mg) corresponds to the peak with GLC retention of 9.8min and Sy134/4 (40mg) to that at 6.4min.

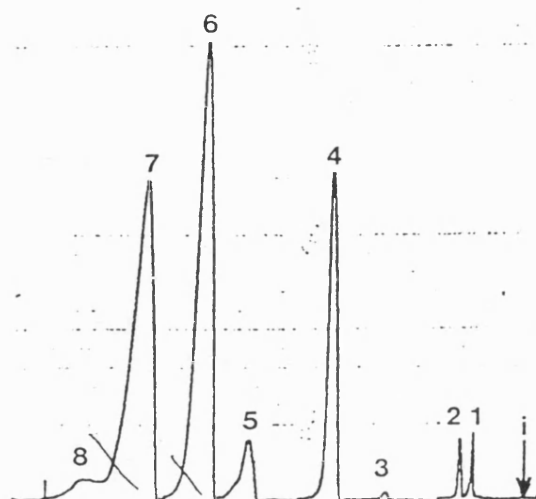


Fig. 2.3: Preparative HPLC profile of Sy134. The point of sample injection is marked "i", and the fractions collected indicated by numbers above the relevant peaks.

NMR and MS data were collected for Sy134/4 and Sy 134/6.

2.3.3 Batch 3 of latex.

250ml of fresh latex was collected into methanol, and stored at 0° before use. The methanol was evaporated under reduced pressure and the remaining soft mass subjected to the partitioning procedure described above. However, instead of using a 90:10 mixture of methanol and water it was decided to use an 80:20 mixture, as a large amount of the sterol-triterpene component of the mixture would partition into the hexane layer as a result of the change in polarity of the methanol phase.

The preparative HPLC apparatus was not available for use at this time and it was decided to use a preparative technique based on centrifugal thin layer chromatography, as this method offered a reduction in time required for the separation as compared with gravity fed column chromatography.

The ether soluble fraction from the third batch of latex (1.9g) was divided into three portions and partitioned on the chromatotron, using a gradient of three mixtures of solvent. The sample was introduced onto the plate which was then eluted with 100ml of toluene:diethyl ether (70:30). The plate was further eluted with 100ml of toluene:diethyl ether:ethyl acetate:methanol (50:20:20:10) followed by 200ml ethyl acetate as a wash to remove any compounds adhering to the plate. The plate was allowed to dry for 30min between the end of a separation and the next sample application to remove any polar solvents that may be present.

The fractions from the plate were analysed by TLC using the solvent system toluene:diethyl ether:ethyl acetate (100:25:25) and like fractions from the three plates were combined. A total of 12 fractions were obtained in this way and coded Sy157.

The fractions from the plate could be classified into two groups, the more polar "yellow" compounds and the less polar "green" compounds. The less polar group were separated from the more polar ones by varying the polarity of the solvents used to elute the plate. Fractions from each separation were assessed by TLC and combined with like fractions within the separation and from other plates.

The solvent system toluene:diethyl ether:ethyl acetate 100:25:25 was found to be effective in separating the constituents of the less polar "green" compounds, with the addition of hexane to reduce polarity where required. The more polar "yellow" compounds could be better separated using mixtures of chloroform and methanol in a proportion of 95:5, although it was found that the addition of hexane to give a solvent system hexane:chloroform:methanol 50:95:5 increased the retention of the compounds on the plate and gave better resolution.

A number of fractions of the less polar compounds appeared to be pure by TLC and these were tested by GLC using the capillary OV-1 column. The final cTLC preparative plate yielded three compounds in pure form, Sy204/1 (1.5mg), Sy204/3 (5.2mg) and Sy204/5 (29mg). These compounds were submitted for NMR and MS. The remaining "green" fractions were mixtures and required further purification.

The more polar "yellow" compounds were more difficult to assess for purity, as they were irreversibly bound to the GLC column. Two fractions from the cTLC preparative plates, Sy181/2 and Sy186/2 appeared pure by TLC and were submitted for MS and NMR.

2.4 Results of fractionation procedures.

Fresh latex was collected into methanol for storage before use. Evans has used this method to store latex for lengthy periods of time without significant deterioration of the product occurring (Schmidt and Evans 1977). After removal of the methanol, the latex was extracted with acetone to separate the smaller organic constituents from the inorganic ones and from the rubber in the latex. The acetone extract was then partitioned to give the hexane soluble, ether soluble and water soluble extracts.

With the first two batches of latex, the hexane soluble fraction was produced by partitioning between hexane and methanol-water (90:10). With the third batch of latex, the methanol-water mixture was changed to 80:20. The result of this was that the bulk of the sterol/triterpenoid fraction was retained in the hexane whereas in the first two batches these compounds were included in the ether-soluble extract and had to be separated from the green and yellow-orange components by chromatography.

From the various isolation procedures, a number of compounds had been purified and were tested by NMR and MS. On the basis of spectral evidence, like compounds were combined so that larger quantities would be available for structure elucidation.

Components of the compounds of intermediate polarity, those which gave R_f values of between 0.49 and 0.35 in the solvent system cyclo hexane:ethyl acetate:diethyl ether (1:1:1) and coloured green on spraying with 60% sulphuric acid, were of interest as it was observed at an early stage from the spectra for these compounds that they were likely to be diterpenoids. However, they were different from most diterpenes from the Euphorbiaceae

by virtue of the lack of any ketonic carbonyl signal in the ^{13}C spectra, and there was the possibility of them being novel. For this reason it was decided to pursue these compounds in preference to the more polar compounds, which coloured yellow to orange on TLC.

The more polar compounds were almost certainly the irritants in the plant. Whilst working with certain fractions of the material it was noticed that irritation of the buccal and nasal mucosa occurred. This phenomenon has been known to occur with phorbol and ingenol type diterpene irritants (Schmidt 1987). It was also noticed that as the irritant fractions were purified on successive silica gel plates or columns, they seemed to lose their irritancy. This is consistent with the behaviour of phorbol-type diterpene esters which undergo transesterification on silica gel, losing activity as they do so. It is not clear whether the non-irritant fractions are equally labile in the presence of some adsorbants.

An interesting observation from the NMR of the more polar compounds is that, although we were unable to obtain the compounds in pure form, it was obvious that the compounds had different ester groups when compared with the non-irritant compounds. The compounds had a greater variety of aromatic esters, such as benzoate, which are commonly found on phorbol-type diterpenes, whereas the non-irritant compounds only seemed to be esterified to either acetate or 2-methyl butyrate. This would suggest that biochemically, the compounds are formed through different pathways.

The isolated compounds were assessed by NMR and MS. On the basis of this, it was established that Sy65/2 from the first batch was the same as Sy134/6 from the preparative HPLC and Sy204/5 from the cTLC of the third batch of latex.

Sy63/1 from the first batch was the same as Sy107/2 from the second and Sy134/4 from the preparative HPLC.

Sy54/8 was the same as Sy124/2 from the second batch and Sy204/3 from the third.

Like samples were bulked in order to maximise sample size of the pure compounds for structure elucidation and biological activity studies.

A number of different isolation procedures were employed in the course of the work. The first compounds to be isolated in pure form gave spectra which were extremely difficult to interpret. Because of the low yields involved (not more than 20mg) and the advantages of having more material for structure elucidation and ultimately for the assessment of biological activity, it was considered necessary to obtain more than one batch of latex to work on. In the course of working on the three batches, many different preparative techniques were used and it is of interest to compare the performance of the different techniques.

Gravity fed column chromatography remained the primary method of fractionation. Although slower than flash column chromatography or centrifugal thin layer chromatography, the resolution of compounds was better than either flash column or cTLC. The method has the advantage of being relatively cheap to operate, with no special apparatus apart from the column required.

Droplet countercurrent chromatography was an especially appealing technique. It offered the advantage of not having an adsorbent which may

react with the compounds being separated, and being a liquid-liquid system meant that recovery of sample should be a simple matter. In the event, the method was disappointing. The apparatus needed extensive modification in order for it to work properly. The modifications and setting up of the apparatus are discussed in chapters 5 and 6. When it did work, recovery of sample from the eluted fractions was not easy, as the solvent systems used employed water as one of the components and this was difficult to remove by evaporation at reduced pressure, below 40°. Choice of solvent systems was limited by flow considerations, and monitoring of fractions by GLC difficult because of the presence of chlorinated solvents in most of the solvent systems employed. The technique takes a long time to effect a separation but this is not an especially serious consideration. A meaningful separation that can be achieved within the space of a week is worthwhile. Separation of two components, Sy107/2 and Sy124/2, was achieved using DCCC in conjunction with column chromatography.

Flash column chromatography is fast, but the resolution is not as good as a conventional gravity column. Preparative thin layer chromatography is effective but poses safety problems. The plates need to be made up and handled in a fairly large space and silica dust is a known hazard compounded by having toxic compounds adsorbed onto it.

Centrifugal thin layer chromatography is extremely fast, with a separation being accomplished within ten to fifteen minutes. However, the resolution is not especially good and the apparatus is expensive.

The method of choice without doubt is preparative HPLC. In this work, establishing a system which worked was not especially time consuming. Once operational, the separation of a reasonably large amount of material was

accomplished within a few working days. The greatest drawback is availability of apparatus and suitable column. For this work the only column available was a silica gel one and it may be that greater resolution may have been achieved had a bonded phase column been employed. In the latter stages of the work, the apparatus was not available for use because of a fault with the equipment and obtaining the correct replacement part proved difficult.

2.5 Structure elucidation.

2.5.1 Sy 215/1.

The compound Sy215/1 was isolated in crystalline form after storage in hexane for 24 months. 12.9mg in total was available for instrumental analysis and structure elucidation.

A 2mg portion was taken for NMR. The ^1H and ^{13}C one-dimensional NMR spectra, together with the two-dimensional COSY, C-H Shift correlation, Long range COSY and chemical ionisation Mass Spectrum are presented as figures.

The ^1H NMR spectrum on first inspection shows a number of similarities to diterpene esters of the phorbol or ingol type. Fig 2.11 shows the structures of phorbol diacetate and ingol tetra-acetate. There are relatively few peaks in the CH_2 region, 1.2ppm - 2.0ppm, and a number of peaks downfield of 3ppm, indicating either unsaturation or proximity to oxygen or other electron withdrawing groups. The compound appears to be esterified and four strong singlets slightly downfield of 2ppm can be observed. These are assigned as acetate methyl groups. There are no peaks in the aromatic region.

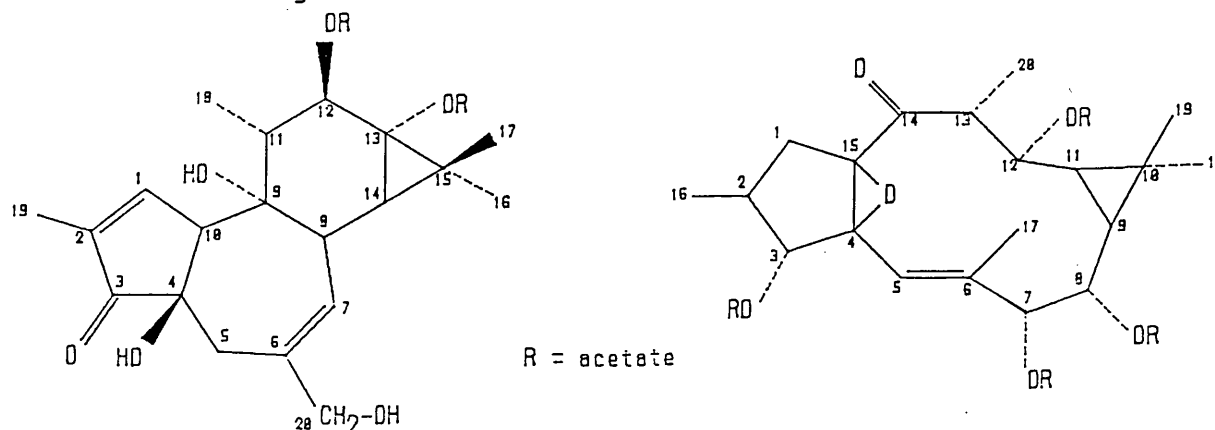


Fig 2.11: Phorbol diacetate and ingol tetraacetate.

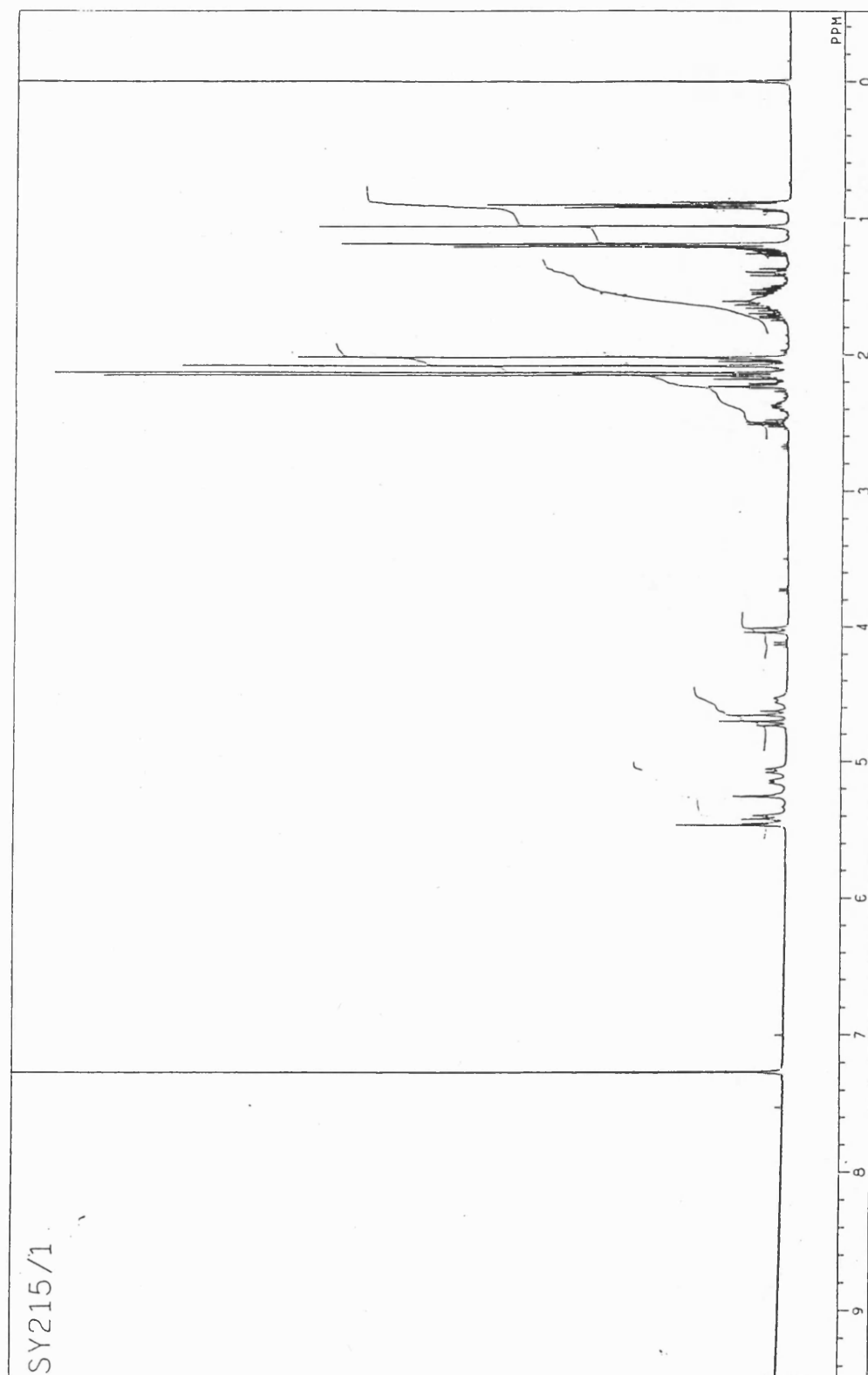


Fig 2.4a 400MHz ^1H NMR spectrum of SY215/1.



Fig 2.4b ^1H NMR spectrum of Sy215/1 expansion.

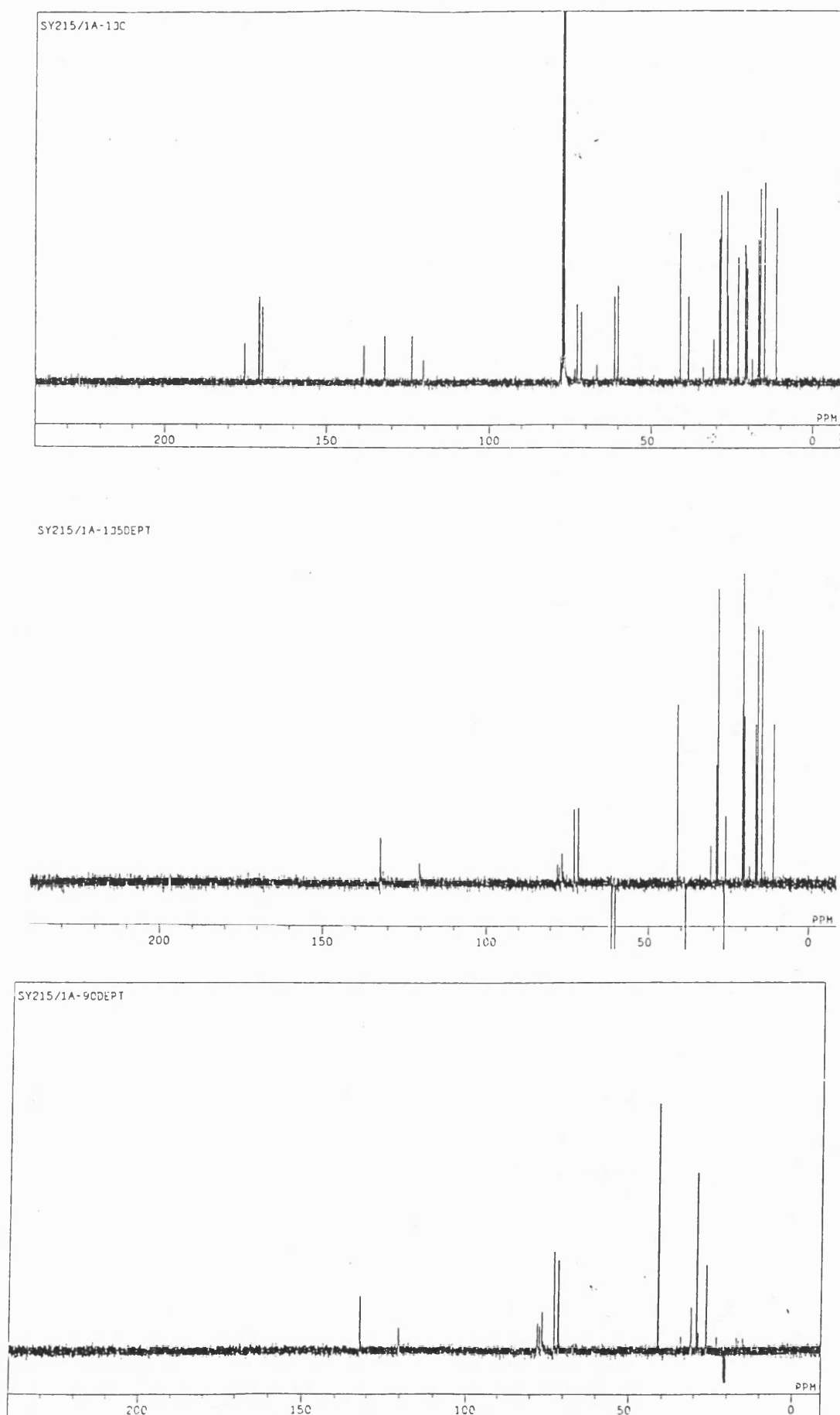


Fig 2.5 67.8MHz ^{13}C NMR spectrum of Sy215/1, with 135 and 90 DEPT plots.

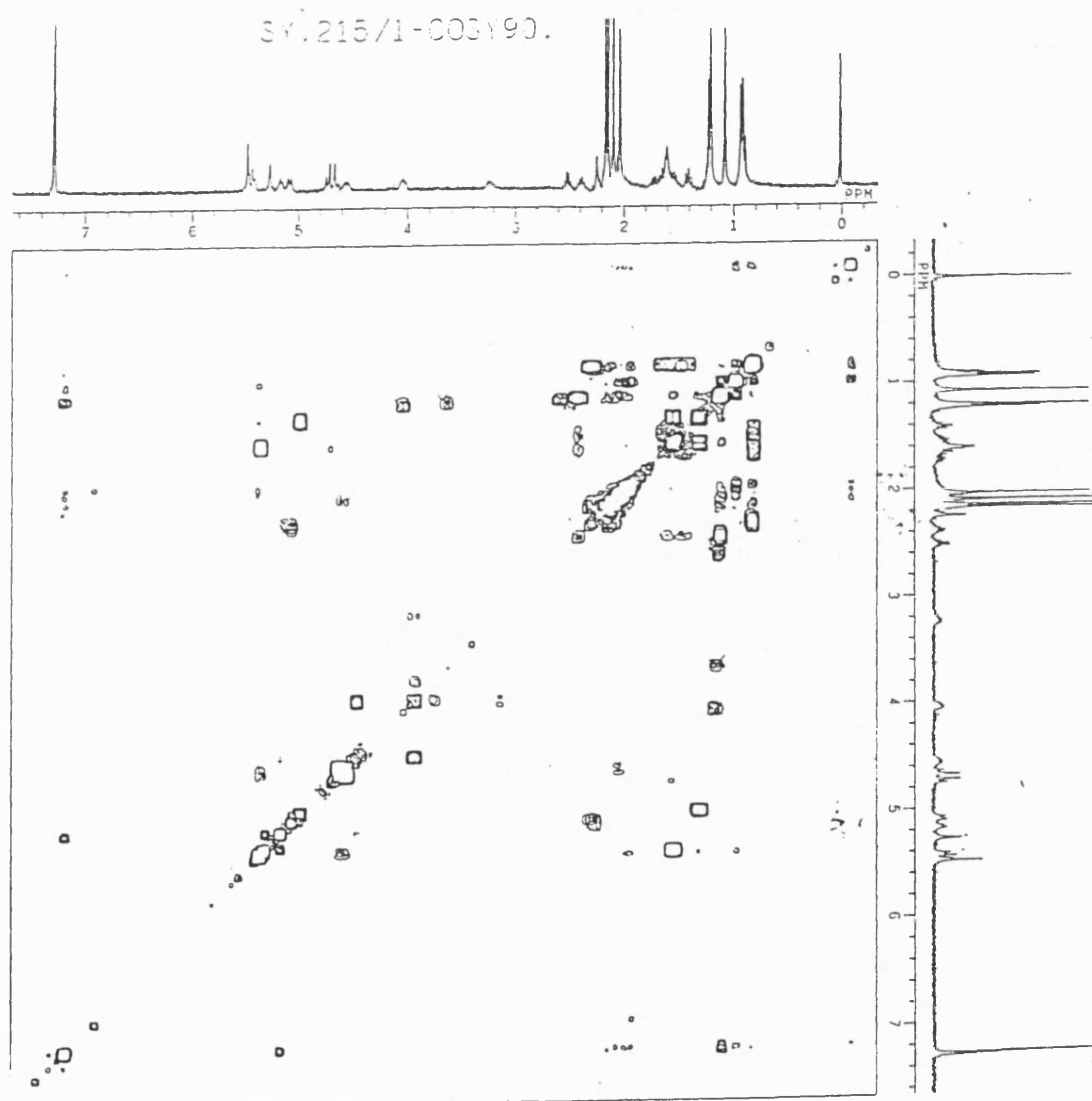


Fig 2.6 Sy215/1 COSY.

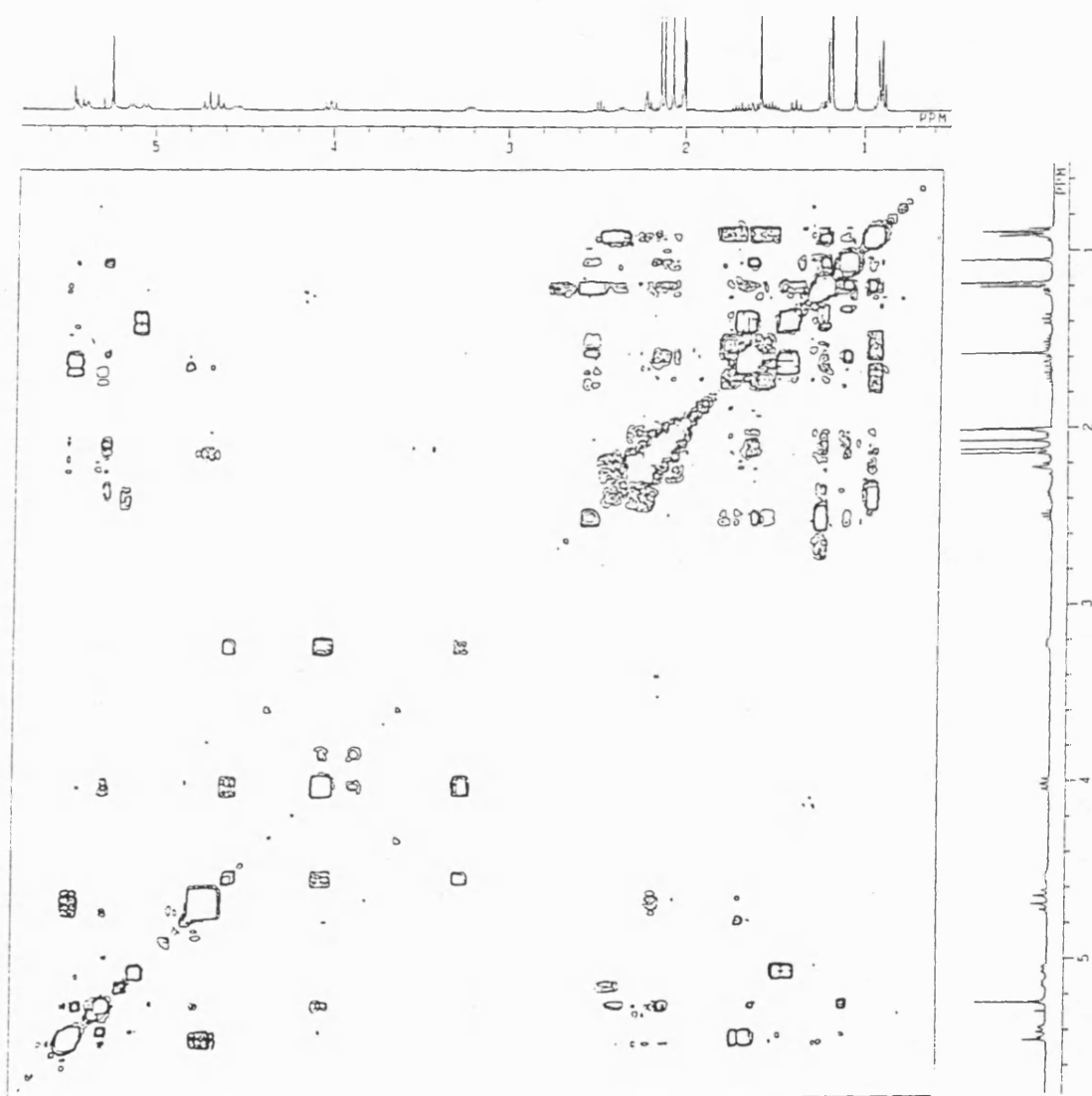


Fig 2.7 Sy215/1 long range COSY.

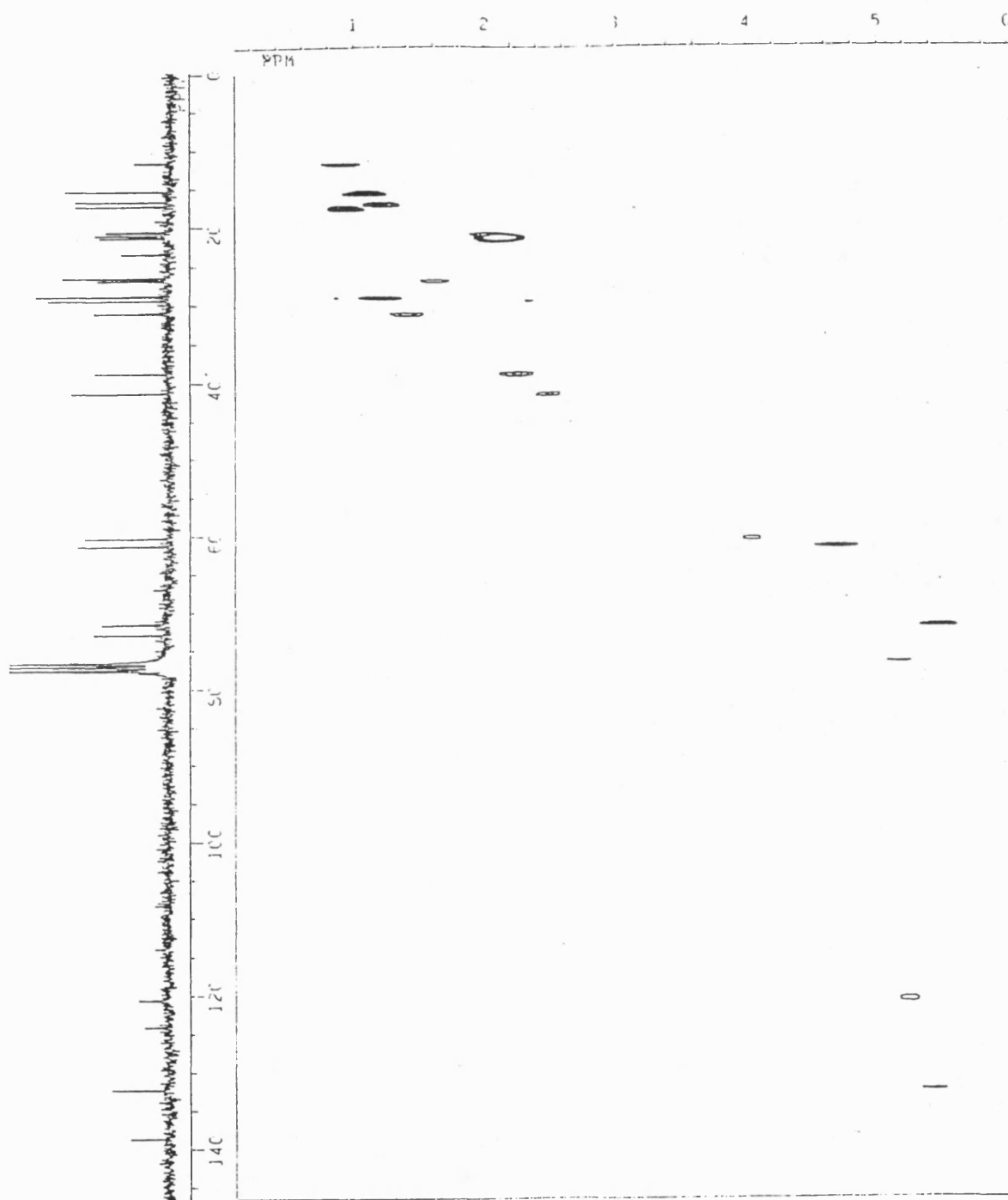


Fig 2.8 Sy215/1 ^{13}C - ^1H shift correlation.

7202C 17 OLIVIER SY215/1 ISO-BUT. C.I.
CAL:CI STA:

03-NOV-87
1118

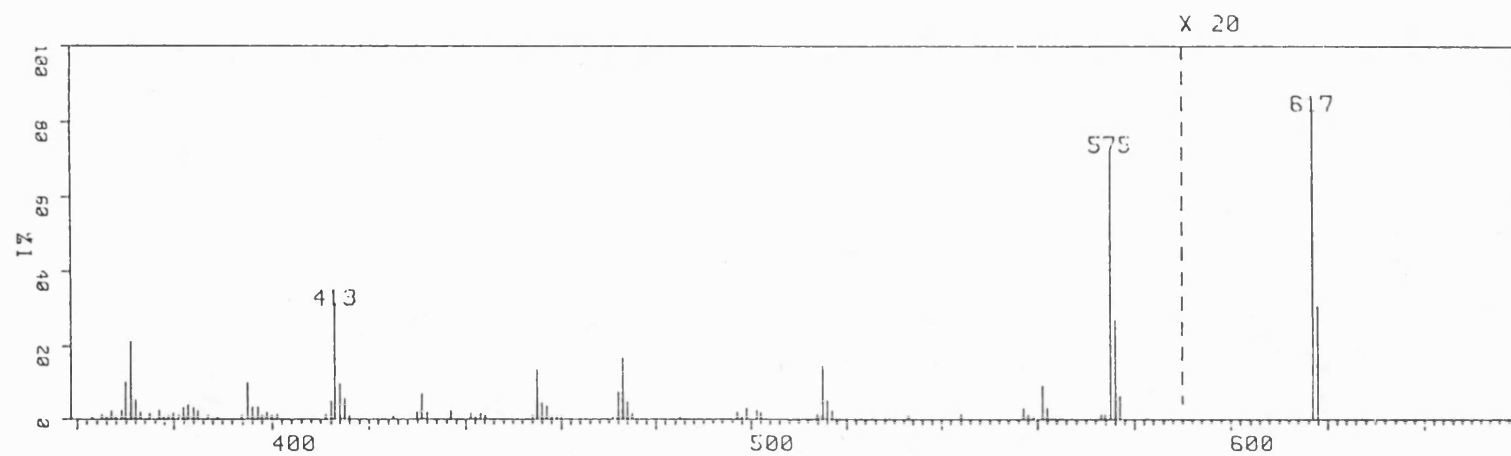
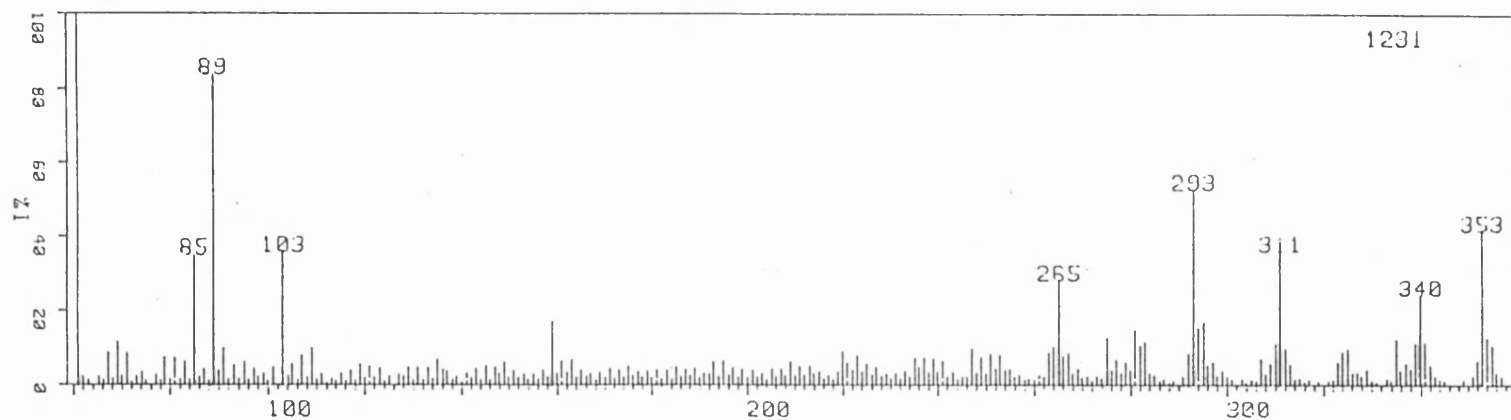


Fig 2.9 SY215/1 Mass spectrum.

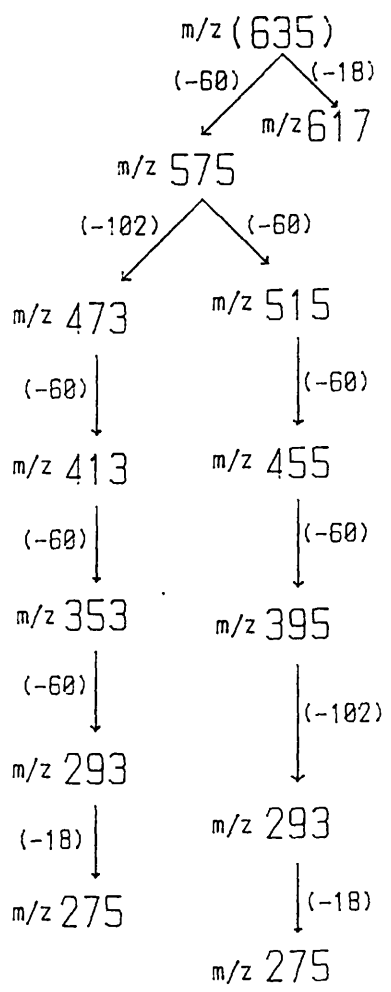


Fig 2.10 Sy215/1 MS fragmentation pattern.

From the COSY plot, Fig 2.6, a number of chains of connectivities can be mapped out:

Segment A: The three proton triplet at 0.91ppm is coupled to the two proton pair of multiplets at 1.53ppm and 1.71ppm, which in turn is coupled to the one proton multiplet at 2.49ppm. This signal is coupled to the three proton doublet at 1.21ppm. This arrangement was assigned to a 2-methyl butyrate ester, which occurs commonly in diterpenoid compounds from the Euphorbiaceae. The assignment of this group is consistent with the loss of a 102 molecular weight fragment in the mass spectrum.

Segment B: The three proton doublet at 0.92ppm is coupled to a one proton multiplet at 2.37ppm. This in turn is coupled to a two proton multiplet at 2.23ppm and a one proton broad doublet at 5.14ppm. A similar arrangement occurs in ingol tetra-acetate and is assigned to the methyl group at position 16 coupled to the single proton at position 2 which is coupled to the geminal protons at position 1 and the single proton at position 3. The downfield resonance of the proton at position 3 suggests proximity to an oxygen containing group as in ingol tetraacetate. No further couplings were observed, suggesting that the adjacent carbon atoms are quarternary.

Segment C: The broad doublet integrating to one proton at 5.07ppm is coupled to the one proton multiplet at 1.39ppm which is coupled to the one proton multiplet at 1.63ppm. This in turn is coupled to the one proton doublet at 5.44ppm. This would appear to be a four-carbon segment with either a double bond or an oxygen containing group at either end. The multiplet at 1.63ppm appears to have a weak coupling to the AB system at 4.64ppm and 4.72ppm, which may be a long-range coupling.

Segment D: The one proton multiplet at 5.25ppm has a very small coupling to the signal at 4.55ppm. It also has a long range coupling to the signal at 5.40ppm. The long-range COSY experiment, set up to reveal

extremely small couplings, shows a coupling to the signal at 4.02ppm.

The ^{13}C spectrum shows four signals in the double bond region, between 120ppm and 140ppm, corresponding to two double bonds. From the ^{13}C - ^1H Shift correlation, the signal at 132.2ppm correlates with that in the ^1H spectrum at 5.44ppm, and the signal at 120.5ppm to that at 5.25ppm. From the ^{13}C DEPT experiments it can be seen that the other two carbon atoms in the double bond region are quarternary. It can be assumed that the chain of connectivities from the COSY experiment (segment C) has a double bond at the end having the proton resonance of 5.44ppm. The second carbon atom of that double bond is quarternary. On the COSY plot a small, long-range coupling can be seen from the signal at 1.63ppm to the AB system at 4.64ppm and 4.72ppm, suggesting that segment C extends through a double bond to a CH_2 group, which is adjacent to an oxygen containing group, either an ester or an alcohol, because of the downfield position of the AB signals. The proton at the other end of the segment resonates at 5.07ppm suggesting that it is adjacent to an alcohol or ester group.

The ^1H and ^{13}C spectra provide evidence for three non acyl methyl groups. One methyl group has been identified as being the one corresponding to position 16 on ingol. There remain two methyl groups in the ^1H spectrum resonating as sharp singlets at 1.06ppm and 1.19ppm. In ingol there are a pair of methyl groups having similar resonances to those at 1.06ppm and 1.19ppm. They form a pair of geminal methyl groups attached to position 10 on the cyclopropane ring. The methyl groups at 1.06ppm and 1.19ppm are assigned as being the geminal pair. The two protons having resonances of 1.39ppm and 1.63ppm are likely to be attached to the carbon atoms forming the base of the cyclopropane ring. The resonances of the protons at 1.39ppm and 1.63ppm, and that of the geminal methyl groups are all in

broad agreement with the corresponding signals associated with the relevant group in ingol. On biosynthetic grounds, a total of five methyl groups would be expected in a diterpene nucleus of the type found in the Euphorbiaceae. This would suggest that two of the methyl groups are in a higher oxidation state. One of these may be the deshielded methylene group revealed as the AB system in segment C.

Segment C would appear to correspond to carbons 8, 9, 10, 11, 12, 18, and 19 of a ring similar to ingol. In addition it would appear that there is a double bond from carbon 12 to 13 and a hydroxymethyl or acyloxymethyl group attached to carbon 13.

From the ^{13}C - ^1H shift correlation the four methyl signals in the ^{13}C spectrum between 20ppm and 21.5ppm correspond to the four acetate methyl groups in the proton spectrum. The carbonyl groups associated with these ester groups resonate at between 169ppm and 175ppm. The presence of four acetates and a 2-methyl butyrate (segment A from the COSY) suggests a highly oxidised nucleus. Of particular interest in the ^{13}C NMR was the absence of any carbonyl signal at around 210ppm. Most of the diterpene ester compounds from the Euphorbiaceae have a ketonic carbonyl group either at position 14 as in ingol or position 3 as in phorbol.

The chemical ionisation Mass Spectrum gives m/z 617 as the largest ion which could be an $[\text{M} + 1]^+$ pseudo-molecular ion. From the NMR data it is apparent that there are five acyl groups. A diterpene nucleus (C_{20}) plus four acetates and a 2-methyl butyrate would give an empirical formula containing 33 carbon atoms and at least 10 oxygen atoms. $\text{C}_{33}\text{O}_{10}$ would need H_{60} to give a molecular weight of 616. This is impossible as the formula would only allow four double bond equivalents. A formula of $\text{C}_{33}\text{H}_{44}\text{O}_{11}$

would give 12 double bond equivalents. Five would be accounted for by the acyl groups. The cyclopentane ring (Segment B), the cyclopropane ring and the assumed macrocyclic ring another three. Two double bonds would bring the total to ten double bond equivalents. The remaining two double bond equivalents could only be an additional two rings, with one being either an epoxide as occurs in ingol, or a cyclic ether as occurs in some of the jatrophone macrocycles and the other a carbocyclic ring. However, the ^1H NMR spectrum shows too many deshielded signals for the latter type of structure (Fig 2.12).

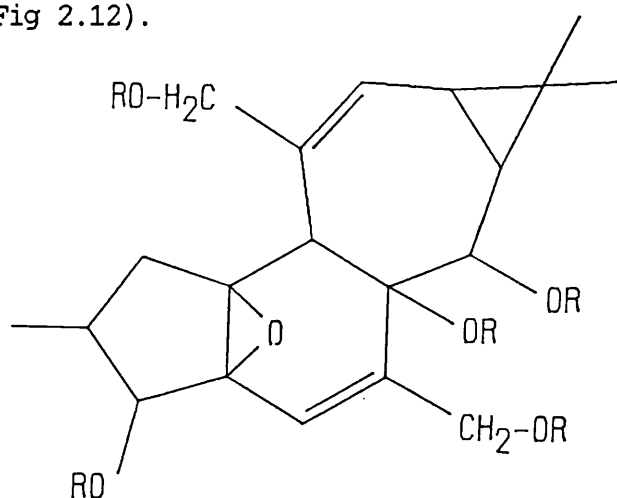


Fig. 2.12: Hypothetical pentacyclic structure for Sy215/1.

The structure and chemical shifts of parts of segments B and C from the COSY experiments are similar to ingol. It could be that the molecule has a basic ring system of the lathyrane type. A third alternative from the mass spectrum is that the empirical formula is $\text{C}_{33}\text{H}_{46}\text{O}_{12}$ which would give a molecular mass of 634. In the C.I.-M.S. spectrum this should give a 635 $[\text{M} + 1]^+$ pseudo molecular ion. An apparent molecular ion of 617 can be accounted for by the loss of water, 635 minus 18. This molecular weight would give 11 double bond equivalents, ten accounted for as above plus one other - possibly an epoxide as in ingol. On this basis the molecule could be of the lathyrane type, with five esterified and one free alcohol.

In the structure elucidation of diterpene esters from Euphorbiaceae, mass

spectral data primarily gives information on acyl substitution. The parent nucleus is normally unstable in the instrument and does not give large fragments. The fragmentation of the esters from the nucleus is usually fairly clear, and, in this instance, the loss of four acetates from 635 to 575, 515, 455 and 395 (loss of 60 in each case) and one 2-methyl butyrate from 395 to 293 (loss of 102) can be observed. In addition, the loss of 102 from 575 to 473, followed by the loss of three 60 mass unit fragments to 413, 353 and 293 can also be seen. The loss of acetate as 60 mass units and 2-methyl butyrate as 102 indicate proximity of the acyl groups to protons, as the esters are lost as free acids. The fragmentation of 635 to 617 is likely to be a minor one, as the peak is only 16% intensity and subsequent fragmentation from 617 is unclear.

If it is assumed that the diterpene has a lathyrane nucleus, then the following partial structure can be put forward (Fig 2.13):

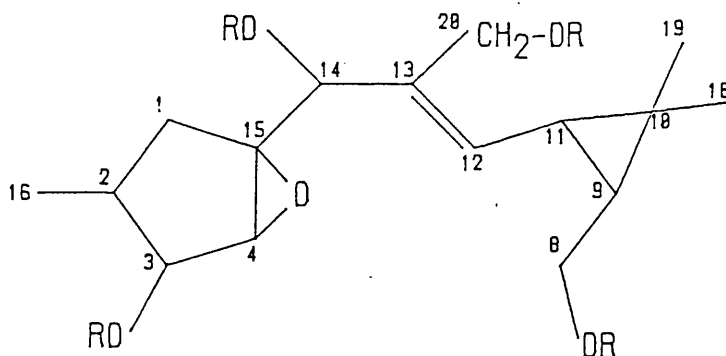


Fig. 2.13: Partial structure of Sy215/1.

It is proposed that carbon 14 is an alcohol rather than a ketone as in ingol. This is then esterified and accounts for one of the acyl groups. The singlet at 5.47ppm in the ^1H NMR is assigned as the proton at 14. This is supported by two small couplings in the COSY experiment to one of the protons at position 20 and to one of the protons at position 1. The long range coupling to the proton at position 20 is further confirmed in the long-range COSY experiment. The positioning of the epoxide group between

carbon atoms 4 and 15 is supported by the termination of the COSY connectivities in segment C and in segment B. It had been concluded in the discussion of segment B that the two carbon atoms at 4 and 15 are quarternary.

There are acyl substituents at position 3 and 8, both of which carry a proton which is deshielded in the ^1H NMR spectrum. The carbon atom at position 20 is oxidised to an alcohol, which may be esterified.

The remaining fragment corresponding to carbon atoms 5, 6, 7 and 17 is less clear. There is a double bond in the fragment composed of one quarternary and one CH carbon. This may be between carbon atoms 5 and 6 or 6 and 7. If it is between carbon atoms 6 and 7, then the fifth acyl group would be attached to position 5, and if between 5 and 6, the acyl substituent would be at 7. In either case, it would be expected that the single proton at position 7 would show coupling to the proton at 8. No coupling is apparent between these two protons. Connolly (1984b) has measured an extremely small coupling (1.8Hz) between the corresponding protons in ingol tetraacetate. The only observed coupling in the COSY from the proton at 8 is to that assigned to position 9, and this is true for the long-range COSY experiment as well. The fragment then comprises an isopropylene group which fits into the ring system in one of two ways. There is an exo-cyclic CH_2 group which is oxidised to an alcohol. The occurrence of a hydroxy group is supported in the ^1H NMR data which shows a broad peak at 3.2ppm which in the COSY is coupled to one of the protons at position 17 (4.02ppm). The wide separation of the geminal proton signals can be attributed to rigidity of the CH_2 group due to hydrogen bonding of the alcoholic proton to another point in the molecule. There is evidence for a free alcohol group in the loss of water in the MS data.

The proposed structure for the compound as elucidated by spectroscopy is one of two. (Fig 2.14.)

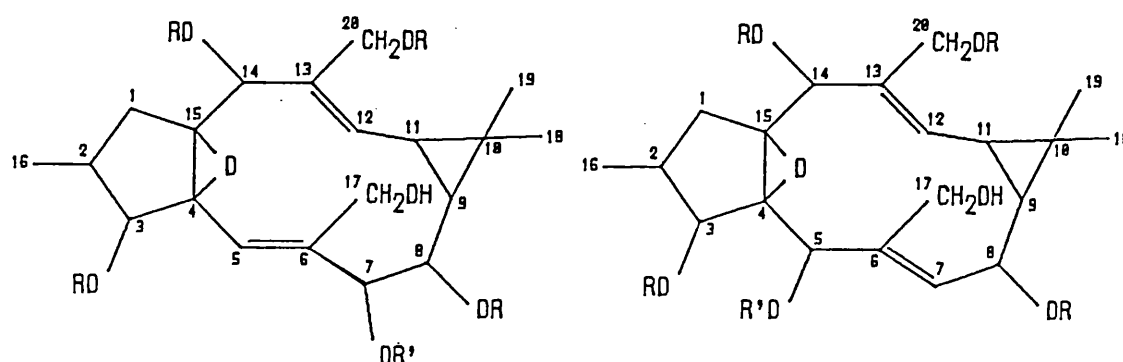


Fig 2.14: Two possible structures for Sy215/1.

The results of the single crystal X-ray study, carried out by Dr K. Molloy and Dr M. Mahon, show that the compound is indeed an ingol type of diterpene with an epoxide ring at position 4,15 and double bonds at 5,6 and 12,13. The acyl groups are attached at positions 3, 7, 8, 14 and 20 with the 2-methyl butyrate at position 7. The x-ray structure, however, showed what seemed to be a cyclopropane ring at position 17, suggesting a compound with a 22 carbon nucleus. This was considered unlikely on biosynthetic grounds and the possibility was ruled out on both x-ray and NMR grounds. On re-examination, the data were found to be more consistent with a hydroxymethyl function in which the oxygen atom was disordered with 50% occupancy in each of two sites. This disorder reflects the existence of two different orientations of the alcohol group, presumably forming two different hydrogen bonds either intra- or intermolecular, within the

crystal structure. The NMR data was consistent with a hydroxymethyl group at this position. A portion of the material was acetylated in pyridine/acetic anhydride. The resulting compound showed an additional sharp singlet in the acetate region and a change in the chemical shifts of the two doublets at 4.02ppm and 4.55ppm. On acetylation the broad peak at 3.2ppm disappeared.

The compound is given the systematic name 3-acetoxymethyl, 1,12-epoxy, 10-hydroxymethyl, 9-(2-methyl) butanoyl, 2,8,13-triacetyl, 6,6,14-trimethyl, tricyclo[10,3,0,0^{5,7}]pentadeca-3,10-diene. We have given the compound the trivial name, based on "Synadenol" for the parent alcohol, 7-(2-methyl) butanoyl, 3,8,14,20-tetraacetyl synadenol, with a numbering system which reflects the fact that it is a 14-dihydro derivative of ingol.

The structure of Sy215/1 is shown in Fig 2.15. An ORTEP diagram of the compound 2.16.

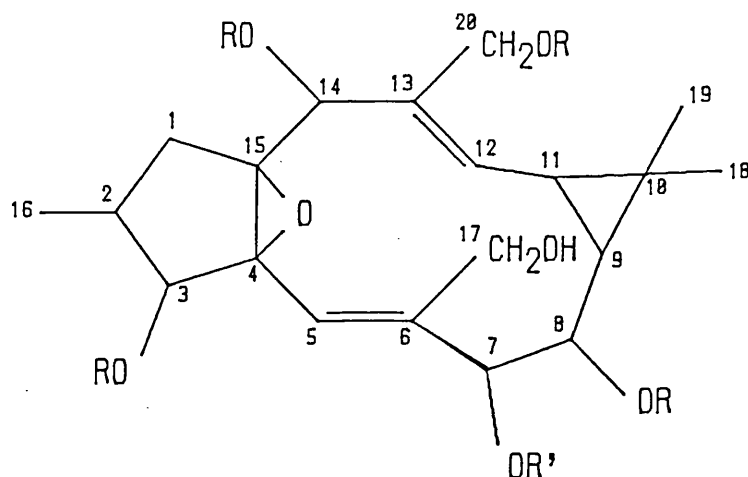


Fig 2.15: The structure of Sy215/1. R= acetate, R'= 2-methyl butyrate

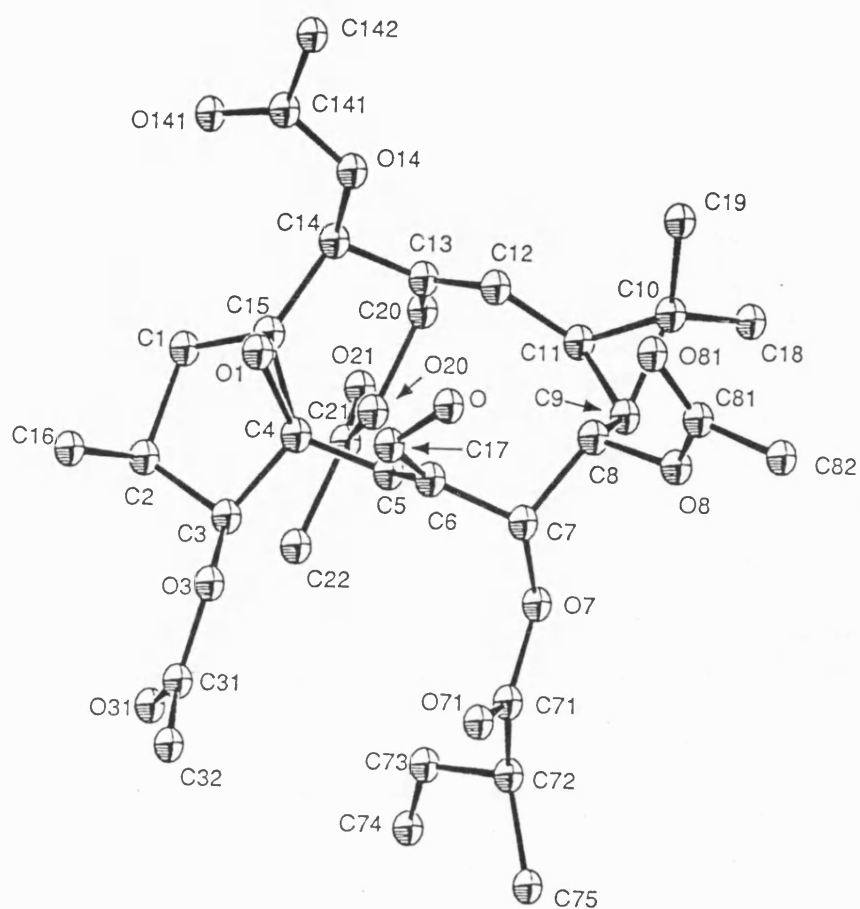


Fig 2.16: ORTEP diagram of Sy215/1.

The ^1H and ^{13}C one dimensional NMR data are presented in Tables 2.1 and 2.2, with assignments.

Table 2.1: Sy215/1 ^1H NMR spectral assignments.

Chemical shift (ppm)	Multiplicity	Coupling constants	Integral	Assignment
0.91	t.	$^3J_{\text{H}4',\text{H}3'} 7.2\text{Hz}$	3	H4'
0.92	d.	$^3J_{\text{H}16,\text{H}2} 7.3\text{Hz}$	3	H16
1.06	s.		3	H19
1.19	s.		3	H18
1.21	d.	$^3J_{\text{H}5',\text{H}2'} 7.2\text{Hz}$	3	H5'
1.39	d.d.	$^3J_{\text{H}9,\text{H}8} 10.8\text{Hz}$ $^3J_{\text{H}9,\text{H}11} 9.3\text{Hz}$	1	H9
1.53	m.		1	H3'
1.63	d.d.	$^3J_{\text{H}11,\text{H}12} 12.2\text{Hz}$ $^3J_{\text{H}11,\text{H}9} 9.3\text{Hz}$	1	H11
1.71	m.		1	H3'
2.02	s.		3	acetate CH ₃
2.07	s.		3	acetate CH ₃
2.12	s.		3	acetate CH ₃
2.15	s.		3	acetate CH ₃
2.23	m.		2	H1
2.37	m.		1	H2
2.49	m.	$^3J_{\text{H}2',\text{H}3'} 13.6\text{Hz}$ $^3J_{\text{H}2',\text{H}5'} 7.2\text{Hz}$	1	H2'
4.02	d.d.	$^2J_{\text{H}17\text{a},\text{H}17\text{b}} 12.8\text{Hz}$	1	H17b
4.55	br.d.	$^2J_{\text{H}17\text{b},\text{H}17\text{a}} 12.8\text{Hz}$	1	H17a
4.64	d.	$^2J_{\text{H}20\text{a},\text{H}20\text{b}} 12.9\text{Hz}$	1	H20a
4.72	d.	$^2J_{\text{H}20\text{b},\text{H}20\text{a}} 12.9\text{Hz}$	1	H20b
5.07	br.d.	$^3J_{\text{H}8,\text{H}9} 10.8\text{Hz}$	1	H8
5.14	br.d.	$^3J_{\text{H}3,\text{H}2} 8.6\text{Hz}$	1	H3
5.25	m.		1	H5
5.40	br.s.		1	H7
5.44	d.	$^3J_{\text{H}12,\text{H}11} 12.2\text{Hz}$	1	H12
5.47	s.		1	H14

Table 2.2: Sy215/1 ^{13}C NMR spectral assignments.

Chemical shift (ppm)	Carbon multiplicity	Assignment
174.9	q	ester carbonyl
170.6	q	ester carbonyl
170.3	q	ester carbonyl
169.4	q	ester carbonyl
138.6	q	6 or 13
132.2	CH	12
124.0	q	6 or 13
120.5	CH	5
77.8	q	4 or 15
77.2	q	4 or 15
76.3	CH	3
72.8	CH	8
71.5	CH	14
66.8		
61.2	CH ₂	20
60.1	CH ₂	17
41.2	CH	2'
38.6	CH ₂	1
30.9	CH	9
29.2	CH	2
28.7	CH ₃	18
26.7	CH ₂	3'
26.3	CH	11
23.3	q	10
21.2	CH ₃	acetate CH ₃
20.9	CH ₃	acetate CH ₃
20.8	CH ₃	acetate CH ₃
20.4	CH ₃	acetate CH ₃
17.1	CH ₃	16
16.5	CH ₃	5'
15.1	CH ₃	19
11.4	CH ₃	4'

2.5.2 Sy65/2, Sy134/6 and Sy204/5.

The compounds, Sy 65/2, Sy134/6 and Sy204/5 were isolated by three different routes. The ^1H NMR spectra for the compounds were identical. Sy65/2 and Sy134/6 were combined to give a final quantity of 80mg.

One-dimensional ^1H and ^{13}C NMR spectra, two-dimensional COSY and ^{13}C - ^1H Shift correlation plots and the mass spectrum are presented as figures.

The ^1H NMR spectrum was identical to that of the acetylated product of Sy215/1. Five acetate methyl signals occur between 2.0ppm and 2.2ppm. A sixth ester group, 2-methyl butyrate, is also present and shows the same chemical shift and splitting pattern as in Sy215/1. From the very close similarities of the NMR spectra, it is assumed that the positions of acetylation for the acetates and 2-methyl butyrate are the same as in Sy215/1.

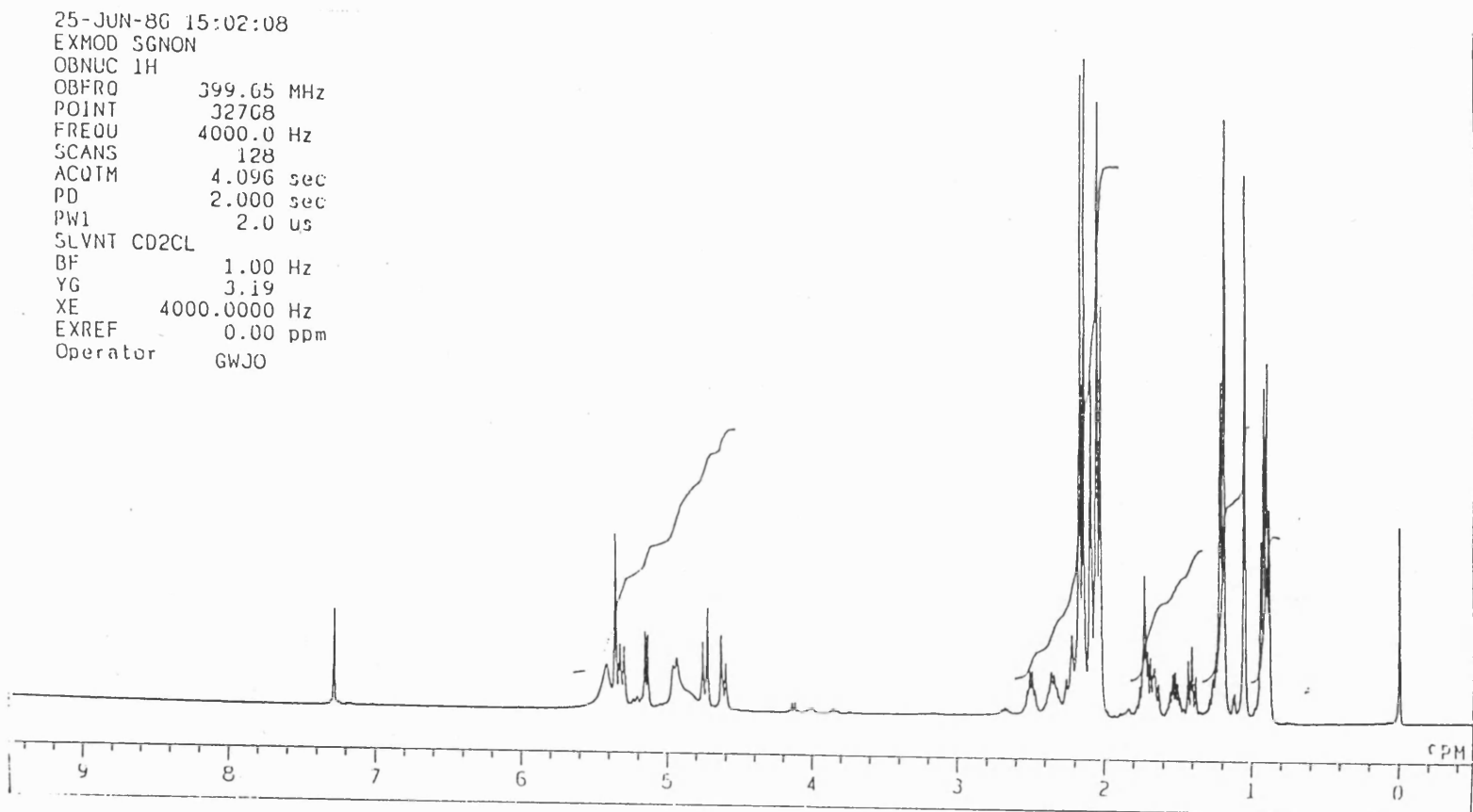


Fig 2.17a 400MHz ^1H NMR spectrum of SY134/6.

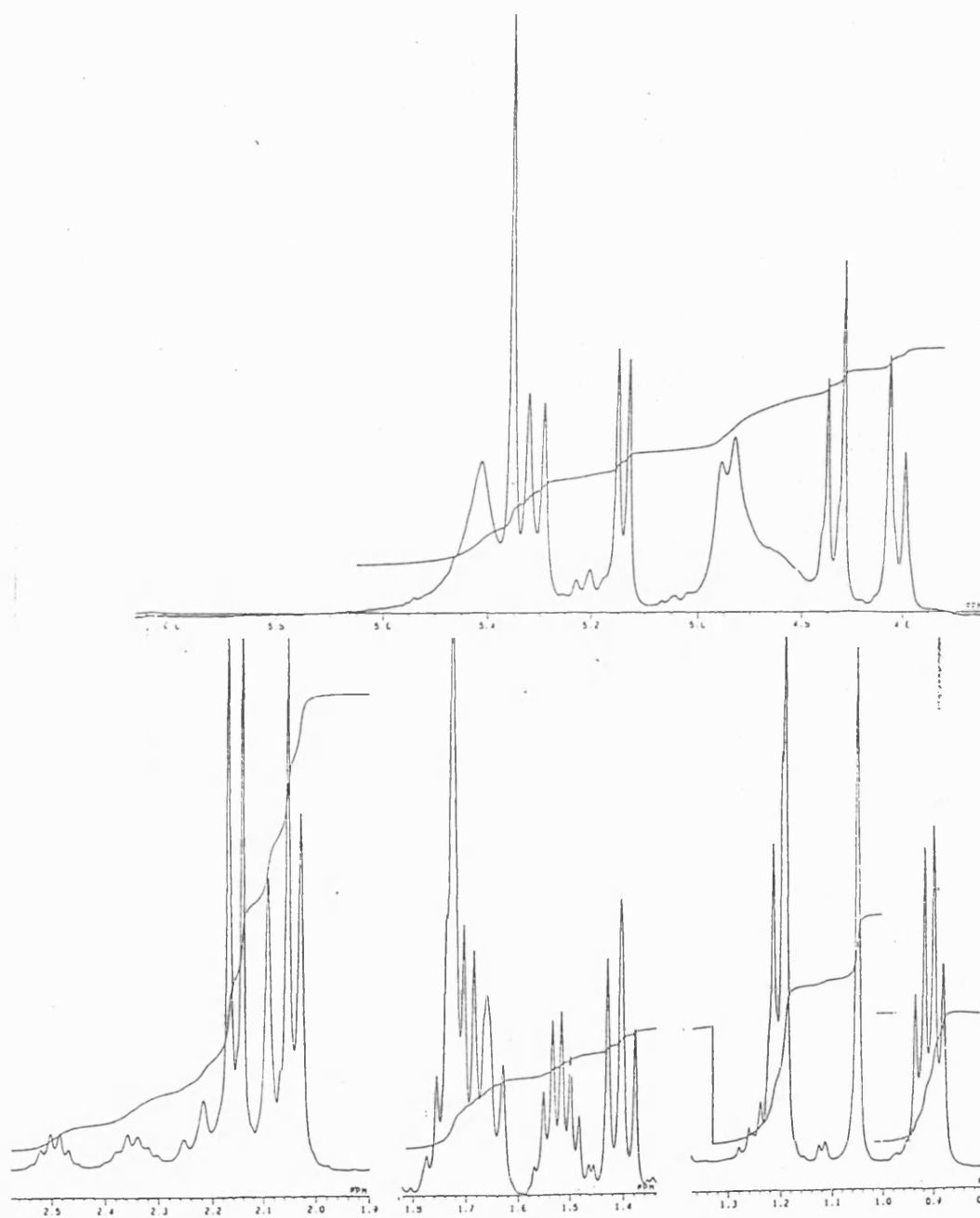
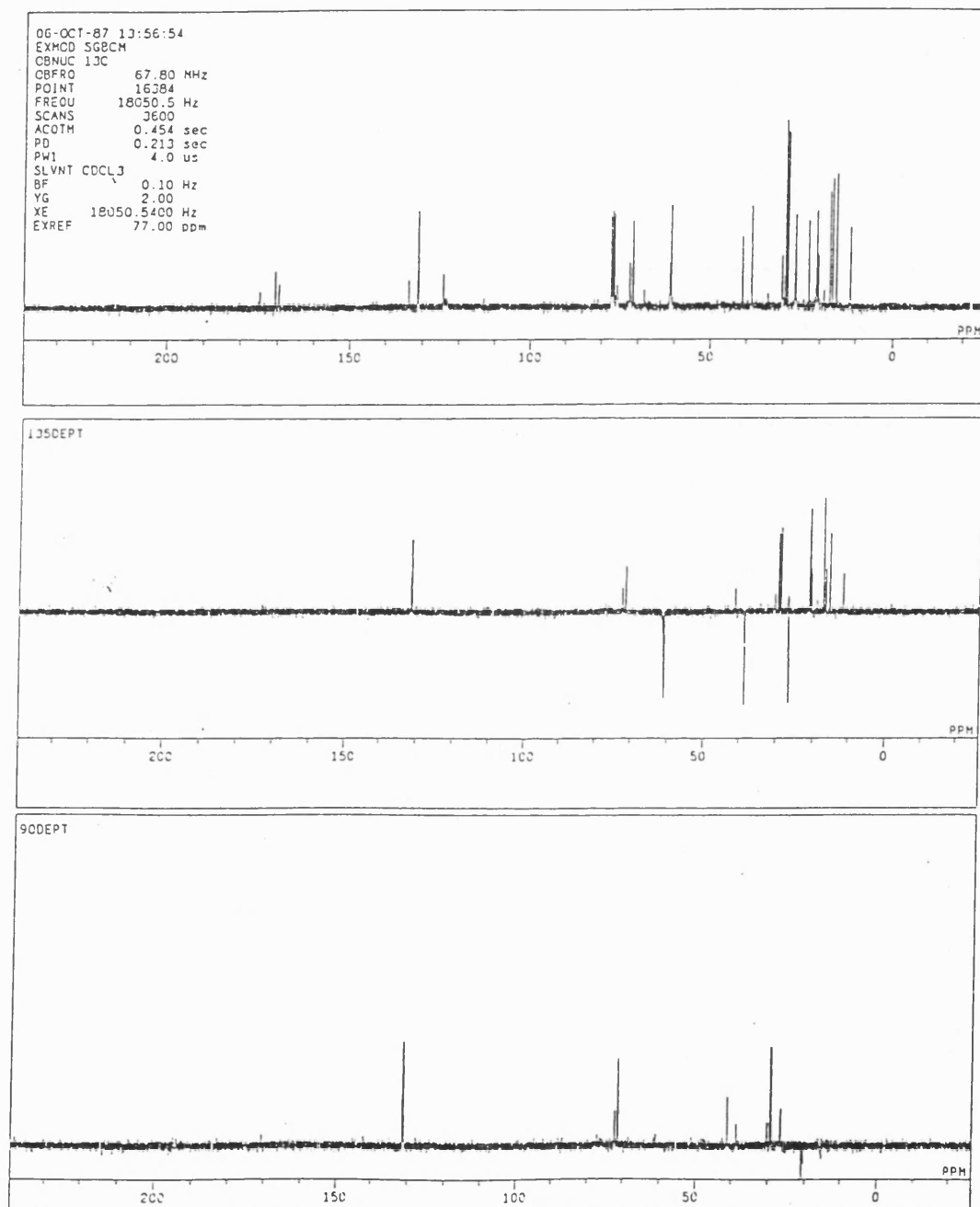


Fig 2.17b ^1H NMR spectrum of Sy134/6 expansion.

SY134/6

Fig 2.18 67.8MHz ^{13}C NMR spectrum of Sy134/6, with 135 and 90 DEPT plots.

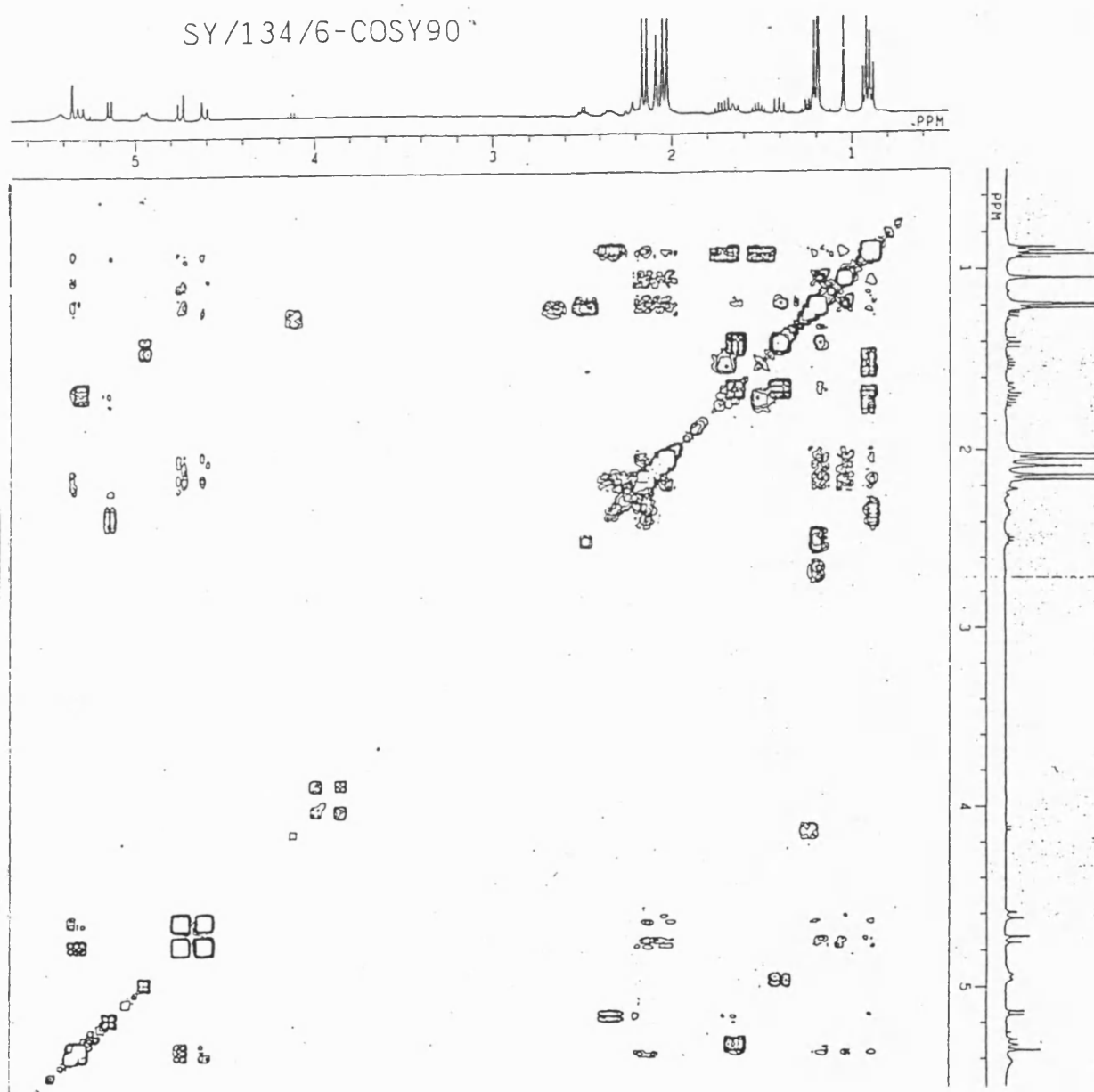


Fig 2.19 Sy134/6 COSY.

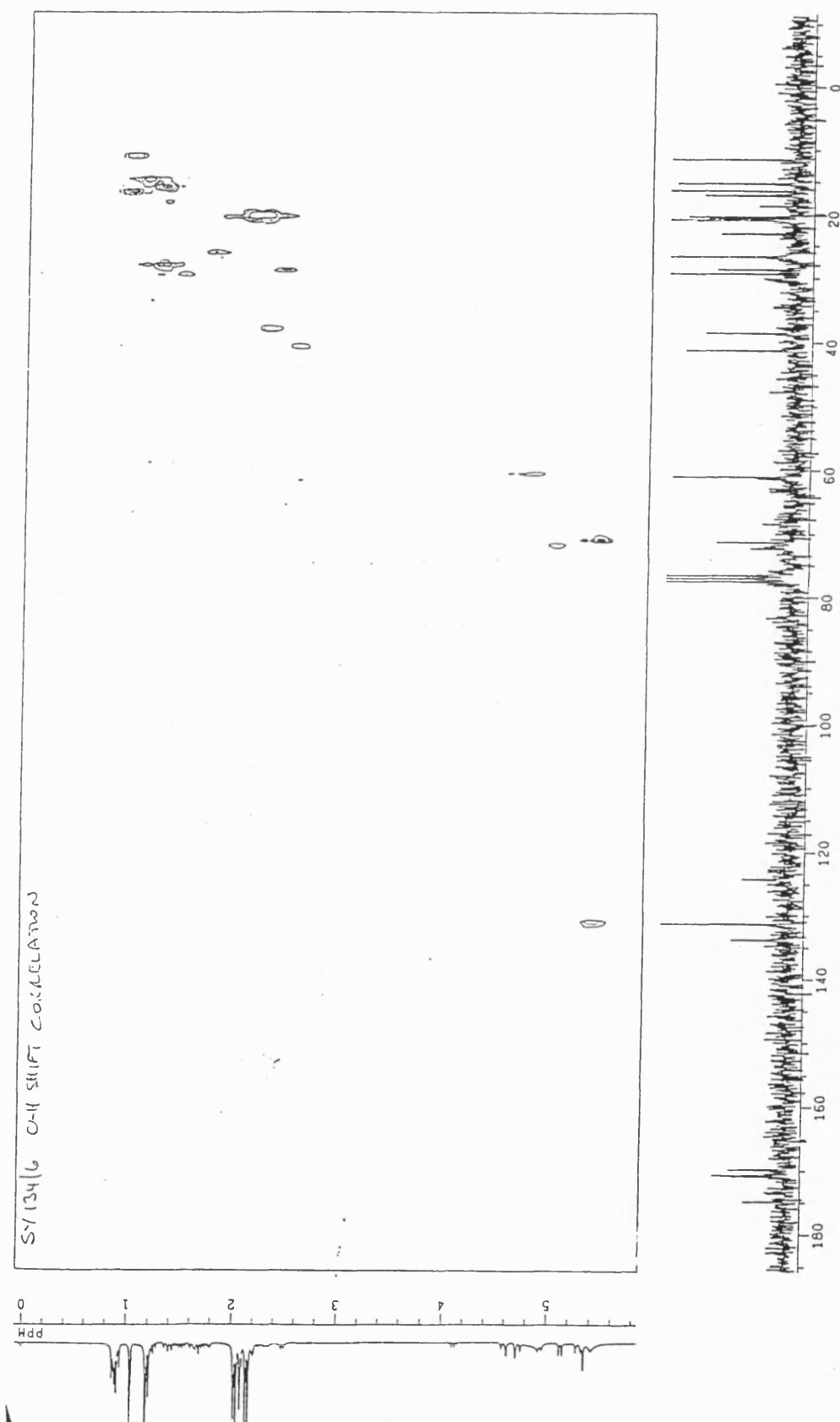


Fig 2.20 Sy134/6 ^{13}C - ^1H shift correlation.

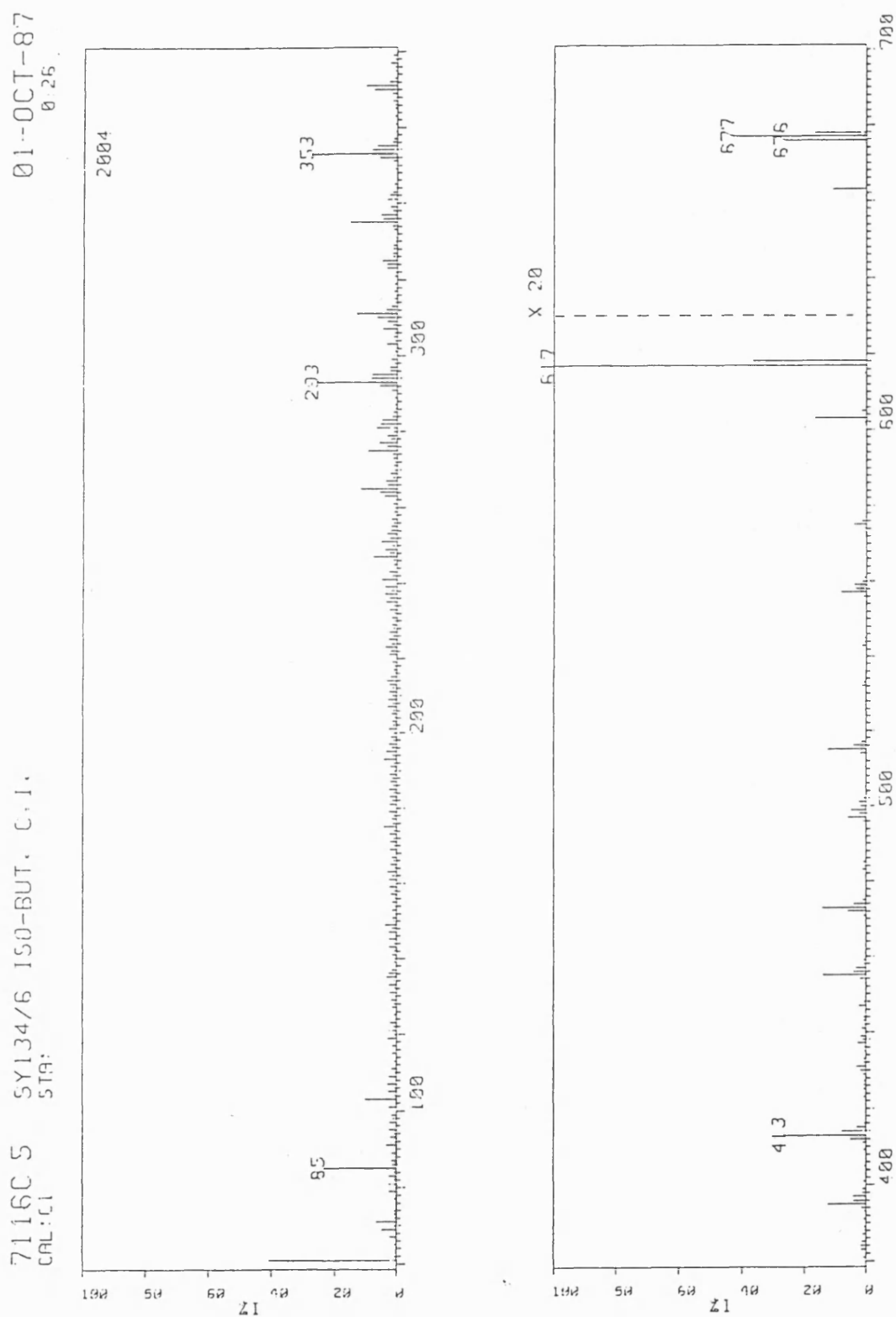


Fig 2.21 Syl34/6 Mass spectrum.

Mass Spec -Sy134/6

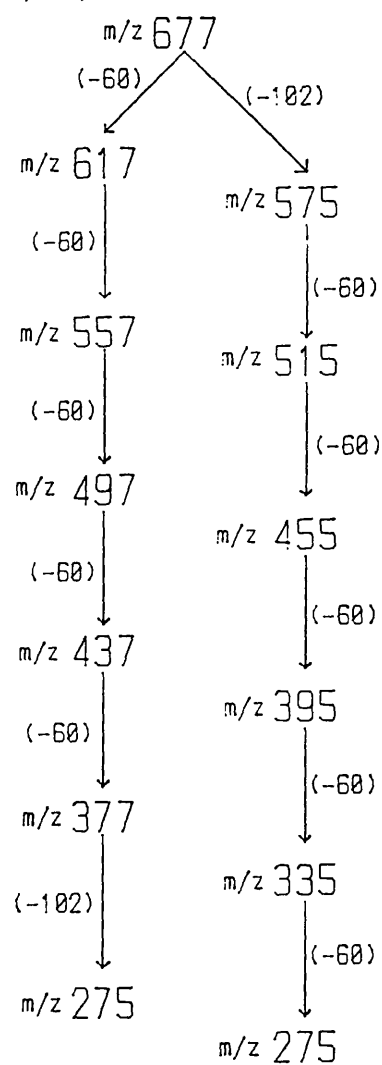


Fig 2.22 Sy134/6 MS fragmentation pattern.

The general assignments and connectivities in the compound are in broad agreement with those made for Sy215/1. The difference in structure between the two compounds lies in the acetylation of the alcohol attached to position 17. In Sy215/1, the CH₂ group formed an AB system with widely separated components possibly due to hydrogen bonding induced rigidity of the CH₂ group. On acetylation of this group, the hydrogen bonding is broken, and the AB signal collapses. The proton spectrum becomes more difficult to interpret as the signals due to the protons at positions 5, 7 and 17 give very broad signals. From the integrals on the spectrum, the broad singlet at 5.42ppm integrates to two protons. The region between 4.8ppm and 5.05ppm integrates to three protons. One of these has already been assigned to the proton at position 8. The other two are likely to be either the AB system at position 17, or the two protons at positions 5 and 7. The unequivocal assignment of these signals is difficult due to poor resolution of the signals and the lack of coupling between them. It might be expected that the two protons at positions 7 and 8 would show some coupling but this is not observed. The same situation exists in Sy215/1.

The mass spectrum is more complex than in Sy215/1. The fragmentation of the compound is given in Fig 2.23. Acetylation of Sy215/1 would give a compound with an empirical formula C₃₅H₄₈O₁₃. This would have a molecular mass of 676, corresponding to twelve double bond equivalents, four accounted for by the ring system, two by the double bonds, and the remaining six by the ester carbonyls. In the chemical ionisation spectrum, a molecular ion of 677 is observed. This is consistent with an [M+1]⁺ pseudo-molecular ion formed by a compound with molecular mass of 676.

A number of fragmentation patterns can be seen in the spectrum (see Fig 2.22). The sequential loss of the ester groups as their free acids, with

acetic acid loss of 60 mass units and 2-methyl butyric acid loss of 102 units gives peaks at 617, 575, 557, 515, 497, 455, 437, 395, 377, 335 and 275. The fragmentation of ester groups does not occur in any specific order. In addition, it would appear that at least one of the acetates is also lost as a 42 mass unit fragment.

The structure of Sy134/6 is shown in Fig 2.23, and the compound is given the trivial name 7-(2-methyl) butanoyl, 3,8,14,17,20-pentaacetyl synadenol.

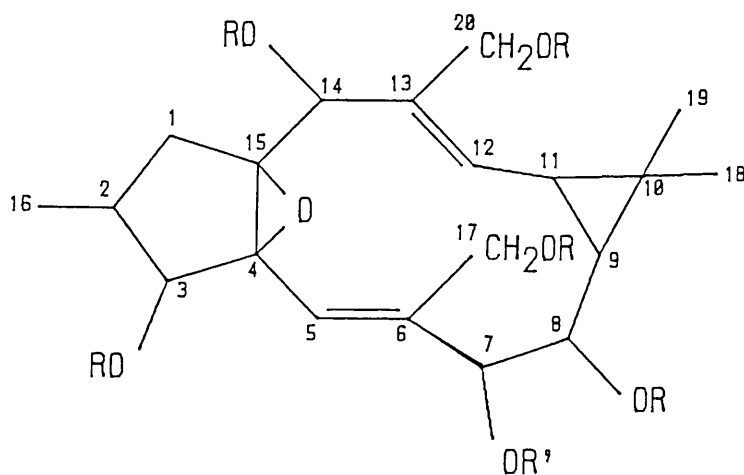


Fig 2.23: The structure of Sy134/6. R = acetate, R' = (2-methyl) butyrate.

Table 2.3: Sy134/6 ^1H NMR spectral assignments.

Chemical shift (ppm)	Multiplicity	Coupling constants	Integral	Assignment
0.91	t.	$^3J_{\text{H}4',\text{H}3'} 7.2\text{Hz}$	3	H4'
0.92	d.	$^3J_{\text{H}16,\text{H}2} 7.2\text{Hz}$	3	H16
1.05	s.		3	H19
1.19	s.		3	H18
1.21	d.	$^3J_{\text{H}5',\text{H}2'} 7.2\text{Hz}$	3	H5'
1.40	d.d.	$^3J_{\text{H}9,\text{H}8} 10.8\text{Hz}$	1	H9
1.52	m.	$^3J_{\text{H}3'\text{a},\text{H}4'} 7.2\text{Hz}$	1	H3'a
1.63	d.d.	$^3J_{\text{H}11,\text{H}9} 9.9\text{Hz}$	1	H11
1.71	m.		1	H3'b
2.03	s.		3	acetate CH ₃
2.05	s.		3	acetate CH ₃
2.09	s.		3	acetate CH ₃
2.14	s.		3	acetate CH ₃
2.17	s.		3	acetate CH ₃
2.23	m.		2	H1
2.35	m.	$^3J_{\text{H}2,\text{H}3} 8.1\text{Hz}$	1	H2
2.49	m.	$^3J_{\text{H}2',\text{H}5'} 7.2\text{Hz}$	1	H2'
4.60	d.	$^2J_{\text{H}20\text{a},\text{H}20\text{b}} 12.6\text{Hz}$	1	H20a
4.72	d.	$^2J_{\text{H}20\text{b},\text{H}20\text{a}} 12.6\text{Hz}$	1	H20b
4.8-5.0	br. m.		3	H8, H17a & b
5.14	d.	$^3J_{\text{H}3,\text{H}2} 8.1\text{Hz}$	1	H3
5.31	d.	$^3J_{\text{H}12,\text{H}11} 12.4\text{Hz}$	1	H12
5.36	s.		1	H5
5.42	br.s.		2	H7, H14

Table 2.4: Sy134/6 ^{13}C NMR spectral assignments.

Chemical shift (ppm)	Carbon multiplicity	Assignment
174.9	q	ester carbonyl
170.8	q	ester carbonyl
170.7	q	ester carbonyl
169.8	q	ester carbonyl
169.7	q	ester carbonyl
133.8	q	6 or 13
131.3	2x CH	5 and 12
124.3	q	6 or 13
77.2	q	4 or 15
76.9	q	4 or 15
76.8	CH	3
72.5	CH	8
71.4	CH	14
68.5	q	unassigned
61.1	2x CH ₂	20 and 17
41.2	CH	2'
38.5	CH ₂	1
30.2	CH	9
29.3	CH	2
28.7	CH ₃	18
26.7	CH ₂	3'
26.7	CH	11
23.1	q	10
21.1	CH ₃	acetate CH ₃
20.9	CH ₃	acetate CH ₃
20.7	CH ₃	acetate CH ₃
20.5	CH ₃	acetate CH ₃
18.0	CH ₃	acetate CH ₃
17.2	CH ₃	16
16.5	CH ₃	5'
15.3	CH ₃	19
11.5	CH ₃	4'

2.5.3 Sy54/8, Sy124/2 and Sy204/3.

The compound Sy204/3, is the same as Sy124/2 and is a more purified form of the sample Sy54/8. NMR and MS data were collected on a 5mg sample.

The one-dimensional ^1H and ^{13}C NMR spectra, the two-dimensional COSY plot and the Mass Spectrum are presented as figures.

On first inspection of the 1-D ^1H NMR spectrum, there are a number of similarities between the compound and Sy215/1 and Sy134/6. Four acetate esters are apparent showing sharp singlets between 2.0ppm and 2.2ppm. An additional ester, 2-methyl butyrate is also present, showing similar chemical shift and splitting patterns as in the previous compounds. The main differences between this compound and the previous two is that one of the methyl singlets previously assigned to position 18 has disappeared. An AB doublet of doublets has appeared at 3.83ppm and 4.01ppm. The chemical shifts for the acetate esters are different, suggesting different positions of esterification.

From the COSY, connectivities linking the protons at positions 8 to 9, to 11 and 12 can be seen. There are further couplings from 12 to a broad multiplet at 1.70ppm, which integrates to three protons, and to the singlet at 5.20ppm assigned to the proton attached to carbon 14. The chemical shift for the multiplet at 1.70ppm is consistent with that for a methyl group adjacent to a double bond.

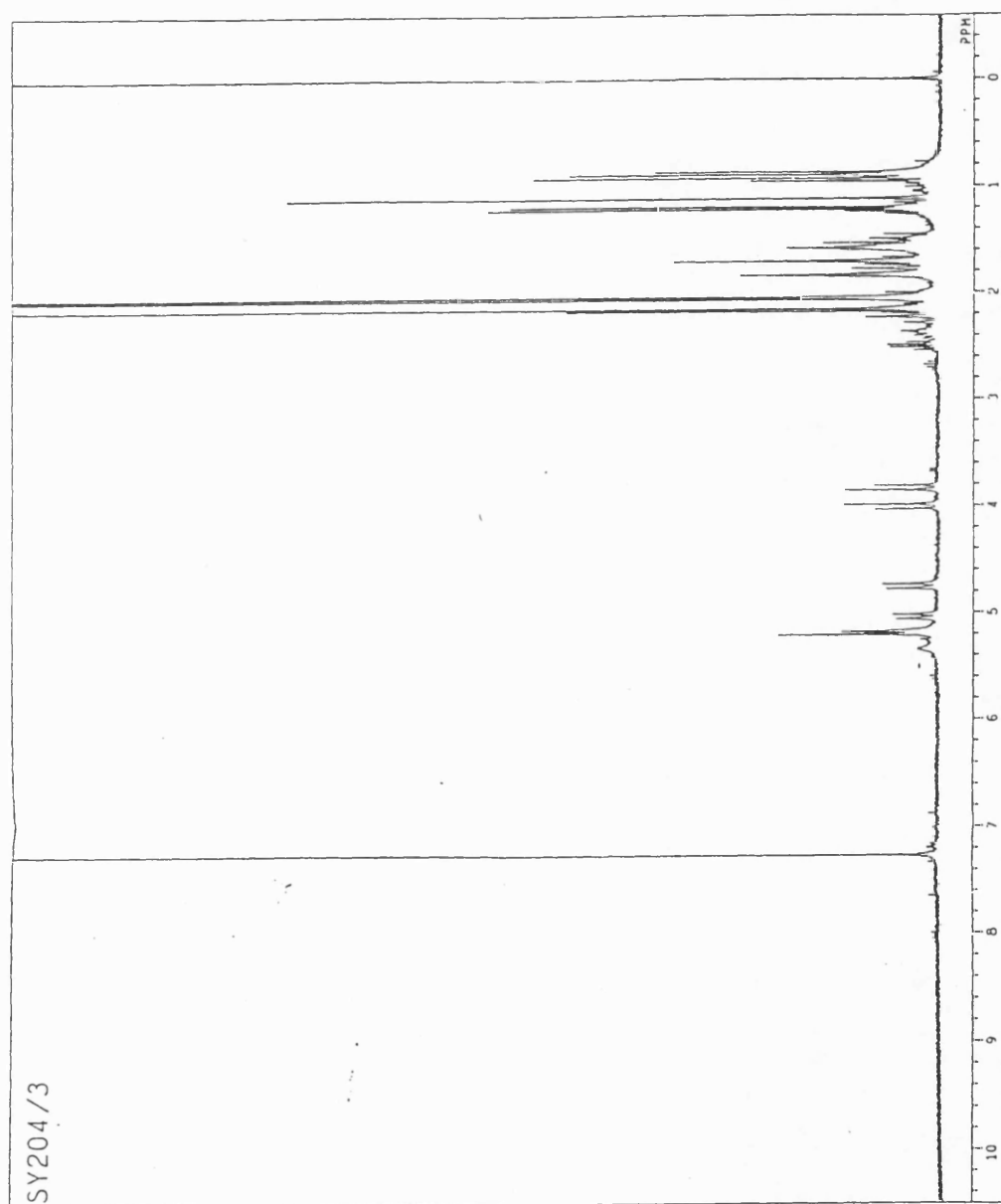


Fig 2.24a 270MHz ^1H NMR spectrum of Sy204/3.

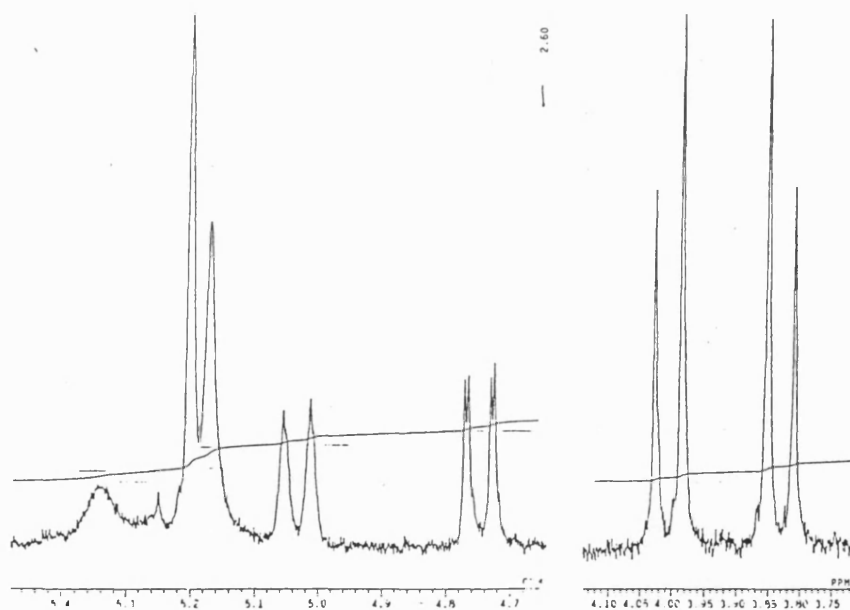
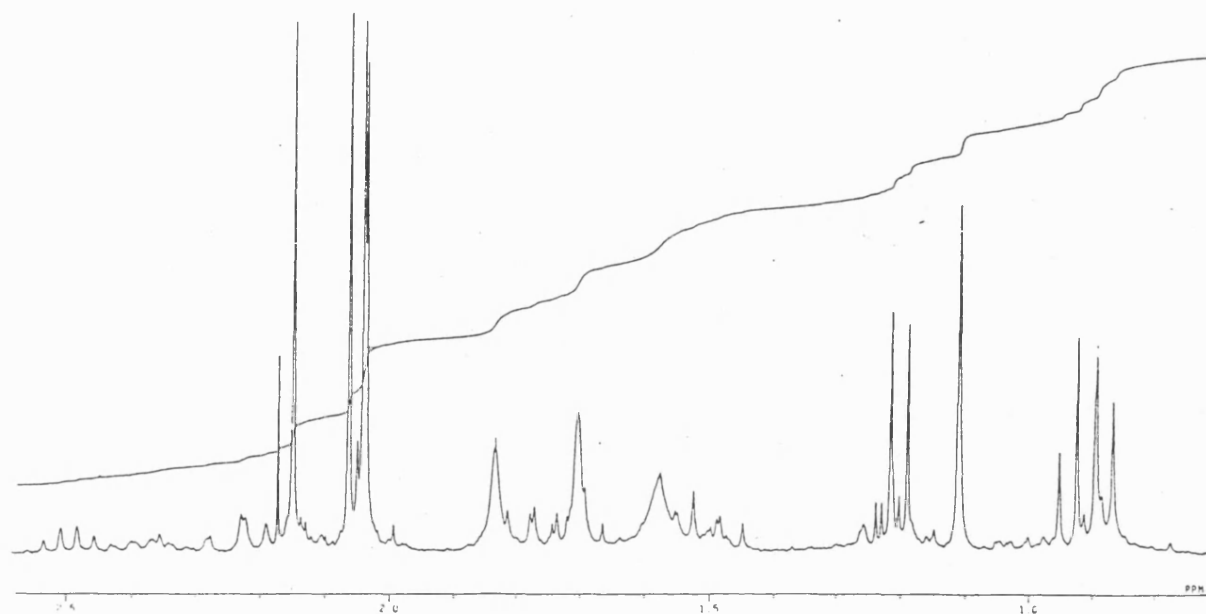


Fig 2.24b ^1H NMR spectrum of Sv204/3 expansion.

SY204/3

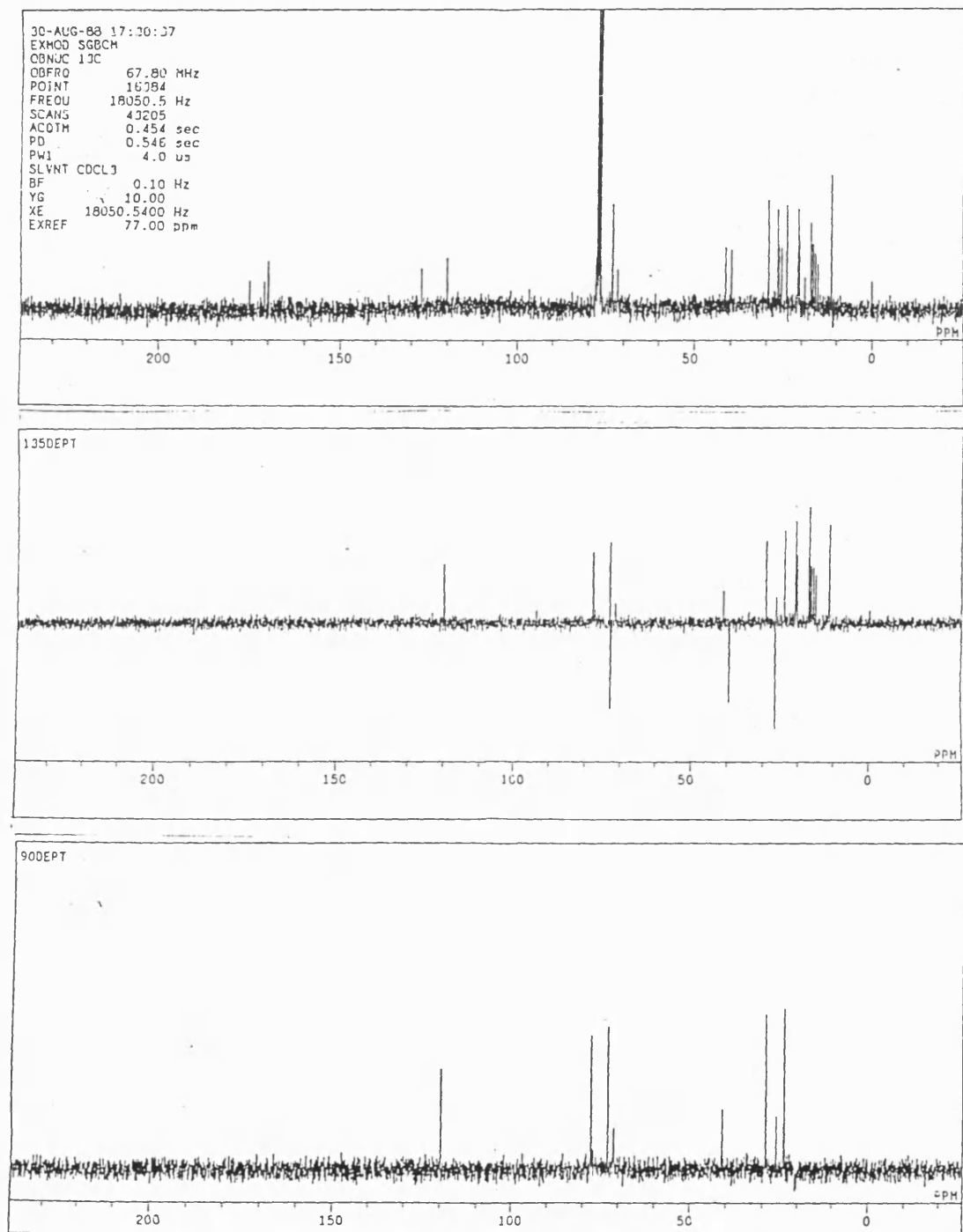


Fig 2.25 67.8MHz ^{13}C NMR spectrum of Sy204/3, with 135 and 90 DEPT plots.

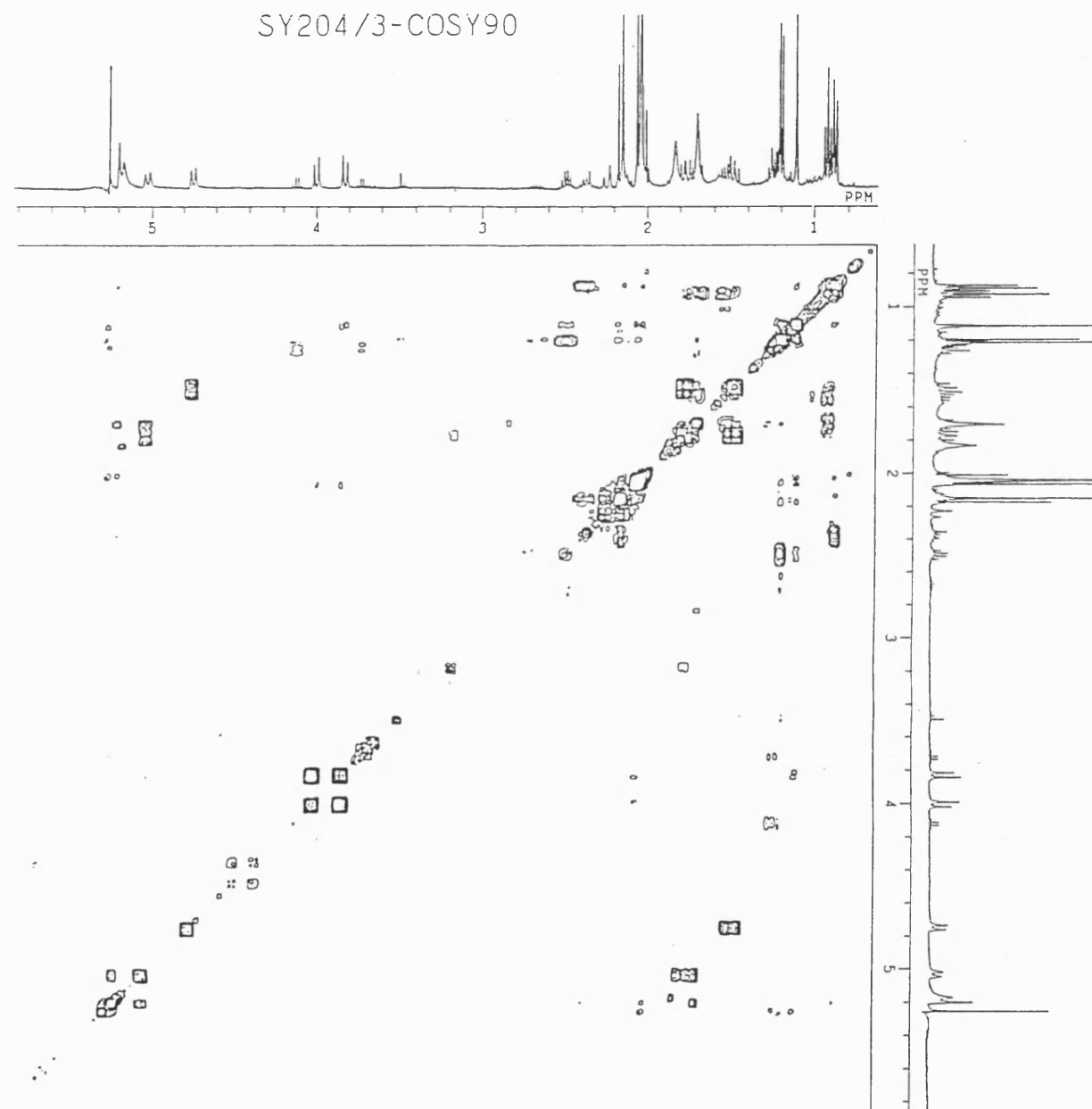


Fig 2.26 Sy204/3 COSY.

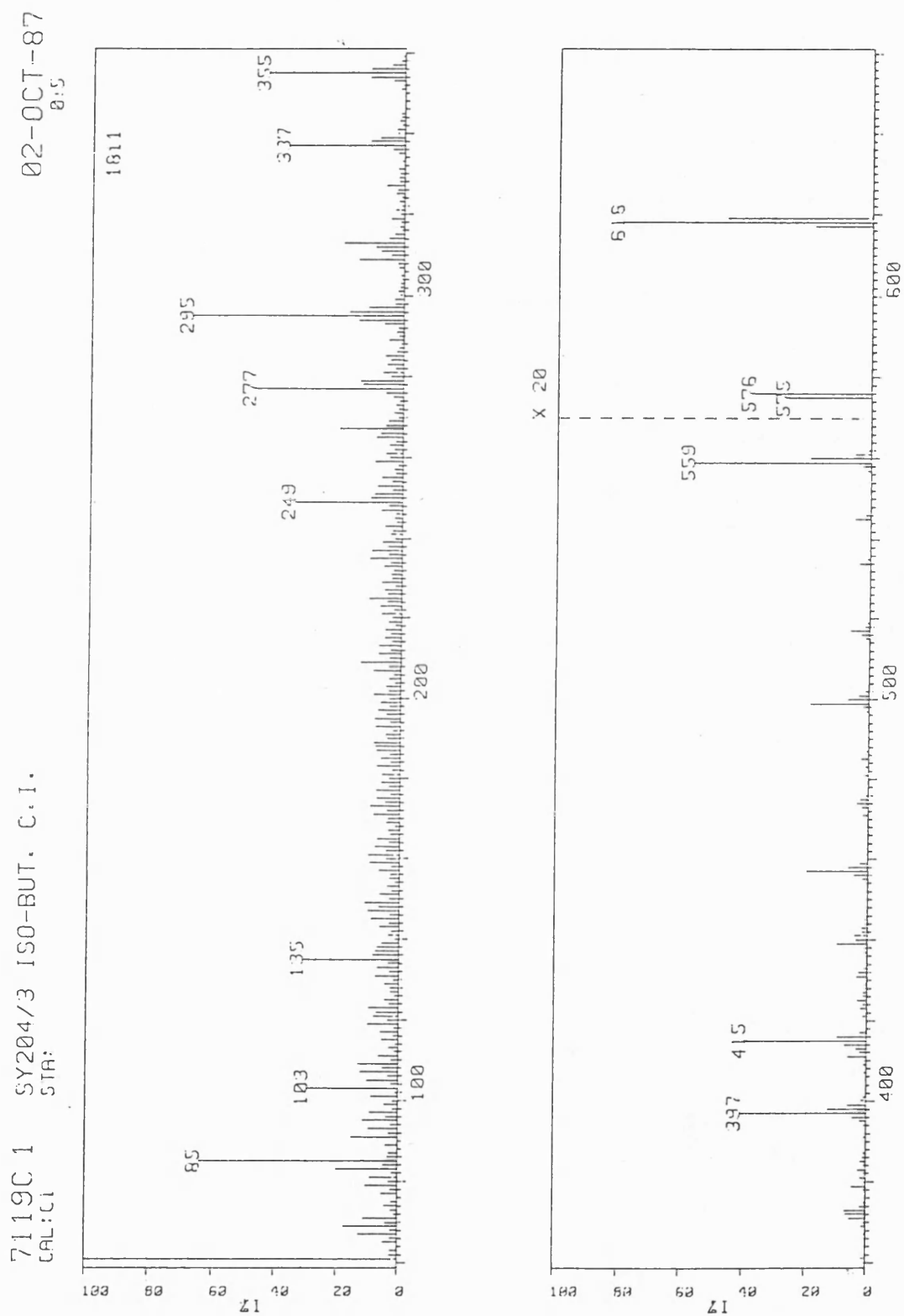


Fig 2.27 Sy204/3 Mass spectrum.

The connectivities between protons at position 1, 2 and 16 are evident as in the previous three compounds. The signal for the proton at position 3 is more obscure, and it may be hidden under the broad singlet at 5.18ppm. There is a small coupling in the COSY between the signal for the proton at position 2 and this signal, which integrates to 2 protons. The broad singlet is assigned to the proton at position 3 and that at position 7.

The detail around positions 5, 6, 7 and 17 is not clear. There is no coupling between the proton at position 7 and that at position 8. There is a coupling between the singlet assigned to position 7 and the multiplet at 1.84ppm. This multiplet integrates to three protons and it is postulated that carbon atom 17 is reduced to a methyl group. There is no signal in the proton spectrum assignable to any protons at position 6 suggesting that carbon 6 is quaternary, with either an alcohol substitution or a double bond to position 5.

Two structures for the compound can be postulated, differing from each other in the arrangement around positions 5, 6 and 7. In the first, Fig 2.29a, there is a double bond between carbon atoms 5 and 6. In the second, Fig 2.29b, carbon atom 6 has an alcohol substituent attached to it and carbon atom 5 is a methylene group. Both of these possible structures have inconsistencies associated with them in the ^{13}C NMR data.

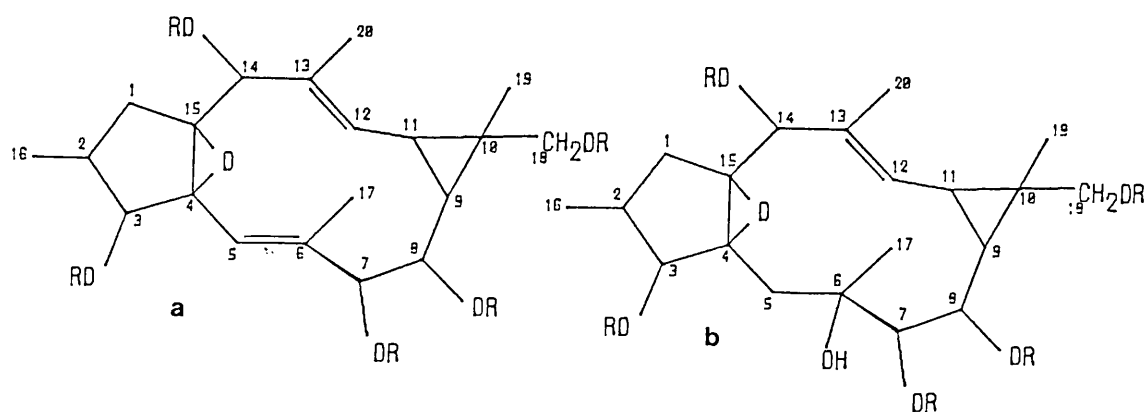


Fig 2.29: Two possible structures for Sy204/3.

The ^{13}C NMR data shows three methylene groups. These are accounted for by carbon atoms 3, 18 and 3' in the 2-methyl butyrate ester. This would appear to rule out the possibility of the structure being that in Fig 2.29b.

If Fig 2.29a is the correct structure, there should be four peaks in the double bond region of the ^{13}C NMR spectrum. In fact, only three peaks are obvious, and one of them is a weak signal possibly due to an impurity. In the ^{13}C spectrum for Sy124/2, Fig 2.30, a broad signal is visible at about 118ppm, and this may be the missing fourth double bond signal.

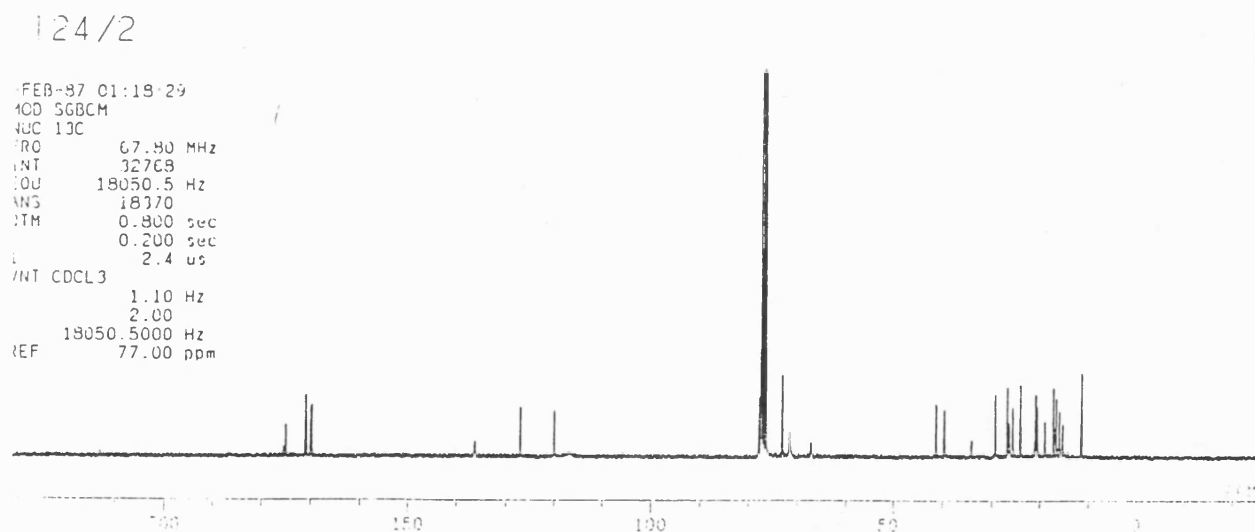


Fig 2.30: ^{13}C spectrum for Sy124/2.

The ^{13}C NMR data does support the reduction of carbon atoms 17 and 20 to methyl groups and the oxidation of carbon atom 18 to a hydroxy-methyl group. There are six methyl signals below 18ppm, corresponding to the six groups in both structures. The methyl signal for carbon atom 18, which in Sy215/1 and Sy134/6 was found at about 28ppm has disappeared.

The mass spectrum could be consistent with both structures. The first structure, Fig 2.29a, would have an empirical formula $C_{33}H_{46}O_{11}$ and would have a molecular mass of 618 units. The molecular ion in the C.I.M.S. spectrum is 619, which would be a pseudo-molecular ion $[M+1]^+$. The second structure, Fig 2.29b, would have an empirical formula $C_{33}H_{48}O_{12}$ which would give a molecular mass of 636. Loss of water as 18 mass units would account for 619 being the highest fragmentation peak in the spectrum. Fragmentation from 619 would be the same for both structures and the fragmentation pattern is shown in Fig 2.31.

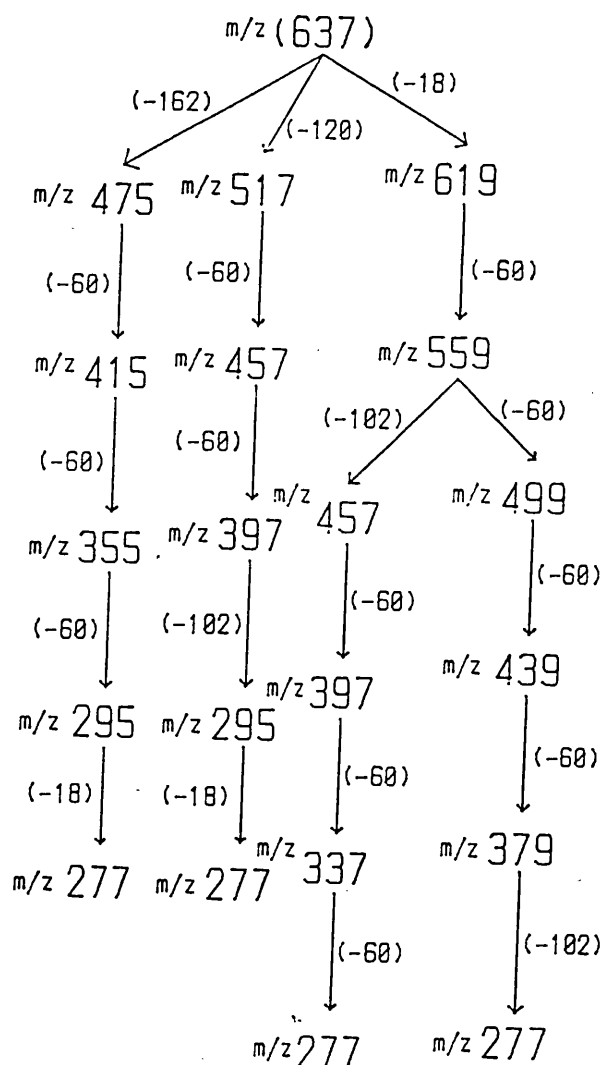


Fig 2.31: Sy204/3 MS fragmentation pattern.

Table 2.5: Sy204/3 ^1H NMR spectral assignments.

Chemical shift (ppm)	Multiplicity	Coupling constants (Hz)	Integral	Assignment
0.90	t.	$^3J_{\text{H}4',\text{H}3'} 7.0\text{Hz}$	3	4'
0.92	d.	$^3J_{\text{H}16,\text{H}2} 7.0\text{Hz}$	3	16
1.11	s.		3	19
1.21	d.	$^3J_{\text{H}5',\text{H}2'} 6.8\text{Hz}$	3	5'
1.49	d.d.	$^3J_{\text{H}9,\text{H}8} 11.1\text{Hz}$ $^3J_{\text{H}9,\text{H}11} 9.6\text{Hz}$	1	9
1.54	m.		1	3'a
1.70	m.		4	20 and 3'b
1.77	m.		1	11
1.84	m.		3	17
2.04	s.		3	acetate CH_3
2.05	s.		3	acetate CH_3
2.07	s.		3	acetate CH_3
2.16	s.		3	acetate CH_3
2.23	m.		2	1
2.38	m.		1	2
2.49	m.	$^3J_{\text{H}2',\text{H}5'} 6.8\text{Hz}$	1	2'
3.83	d.	$^2J_{\text{H}18\text{a},\text{H}18\text{b}} 11.3\text{Hz}$	1	18a
4.01	d.	$^2J_{\text{H}18\text{b},\text{H}18\text{a}} 11.3\text{Hz}$	1	18a
4.75	d.d.	$^3J_{\text{H}8,\text{H}9} 11.1\text{Hz}$ unknown coupling 1.7Hz	1	8
5.03	d.	$^3J_{\text{H}12,\text{H}11} 11.4\text{Hz}$	1	12
5.18	br.s.		2	3,7
5.20	s.		1	14
5.33	br.s.		1	OH or 5

Table 2.6: Sy204/3 ^{13}C NMR spectral assignments.

Chemical shift (ppm)	Carbon multiplicity	Assignment
175.1	q	ester carbonyl
171.1	q	ester carbonyl
170.0	q	ester carbonyl
169.8	q	ester carbonyl
136.3	q	6 or 13
127.2	q	6 or 13
120.1	CH	12 or 5
77.9	CH	3,8 or 14
77.2	q	4 or 15
74.0	q	4 or 15
73.2	CH	3,8 or 14
73.1	CH ₂	18
71.6	CH	3,8 or 14
41.4	CH	2'
39.7	CH ₂	1
30.9	q?	
29.7	q?	
29.4	CH	2
27.8	q?	
27.4	q?	
26.8	CH ₂	3'
26.4	CH	11 or 9
25.6	q	10
24.1	CH	11 or 9
21.0	CH ₃	Acetate CH ₃
20.9	CH ₃	Acetate CH ₃
20.8	CH ₃	Acetate CH ₃
20.5	CH ₃	Acetate CH ₃
19.0	q?	
17.2	CH ₃	16
16.9	CH ₃	17 or 20
16.6	CH ₃	5'
15.9	CH ₃	17 or 20
15.2	CH ₃	19
11.4	CH ₃	4'

2.5.4 Sy 63/1, Sy107/2 and Sy134/4.

The fractions Sy63/1, Sy107/2 and Sy134/4 had identical NMR spectra and were combined. A total mass of approx. 50mg was obtained. MS and NMR data were collected for the sample, and the one-dimensional ^1H and ^{13}C spectra, two-dimensional COSY plot and the Mass Spectrum are presented as figures.

On first inspection of the ^1H spectrum, the compound has many points of similarity with both Sy215/1 and Sy134/6. Four acetate peaks are to be seen between 2.0ppm and 2.2ppm, and the familiar splitting pattern for the 2-methyl butyrate ester. The sharp singlet at 5.40ppm and the doublet at 5.29ppm are reminiscent of the resonances for the protons at position 14 and 12 in Sy215/1. The geminal methyl singlets at 1.05ppm and 1.18ppm are similar to the other two compounds.

From the COSY the connectivities between protons on positions 1 to 2, 2 to 16 and 2 to 3 can be seen, as in the two previous compounds. Similarly, the chain from positions 8 to 9 to 11 to 12 can be seen. Long-range couplings from position 20 to 14 and 14 to 1 are evident. There is no evidence of long-range coupling from 12 to 20 as there was in Sy215/1 and Sy134/6.

The cyclopentane ring system seems unaltered, with a methyl doublet at 0.89ppm (position 16), a multiplet at 2.23ppm (protons at position 1), a multiplet at 2.37ppm (proton at position 2) and a multiplet at 5.19ppm (proton at position 3), and the connections on the COSY can be mapped out.

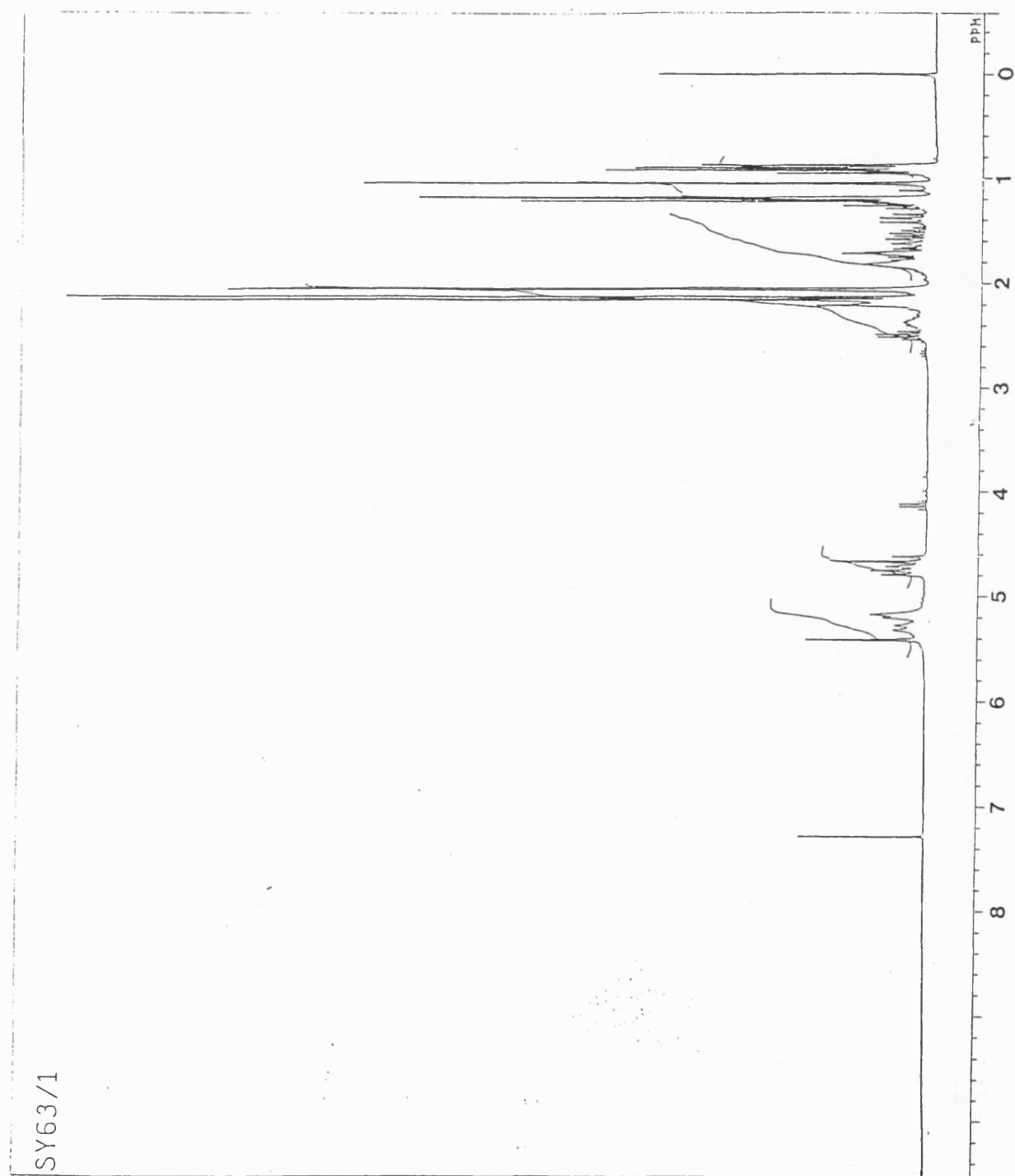


Fig 2.31a 270MHz ^1H NMR spectrum of Sy134/4.

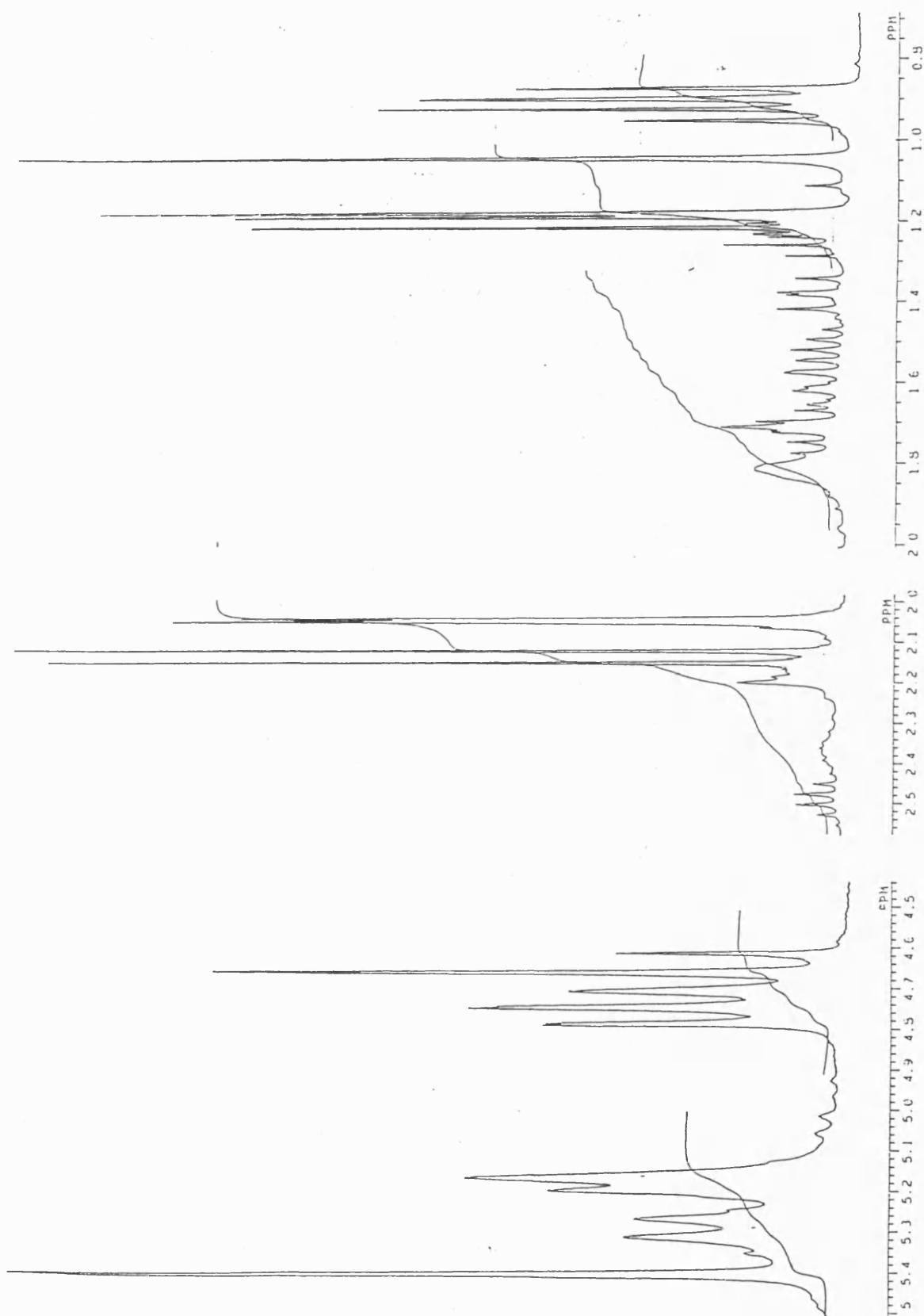


Fig 2.31b ^1H NMR spectrum of Syl34/4 expansion.

SY-134/4

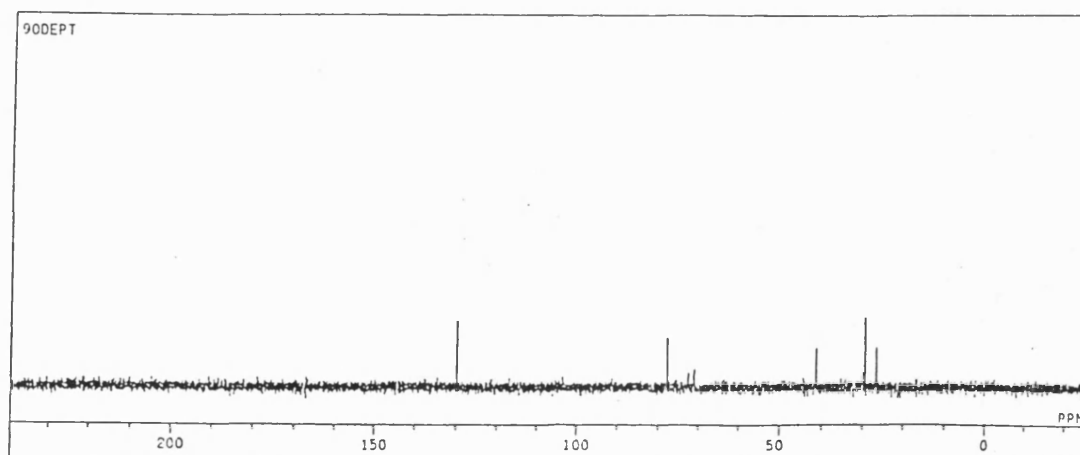
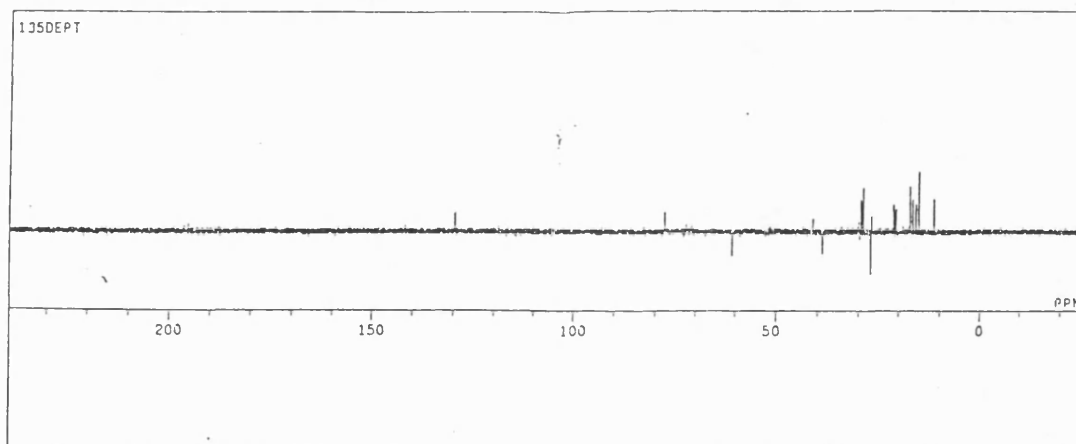
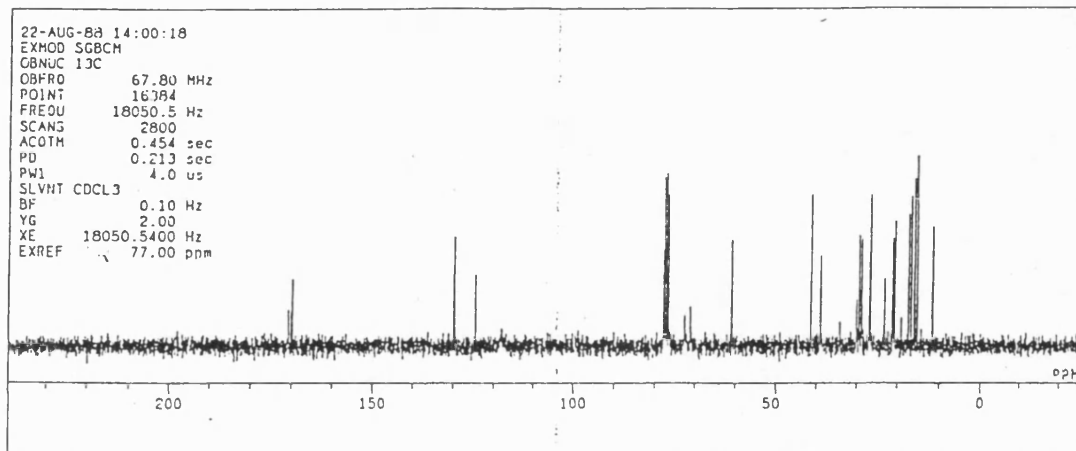


Fig 2.32 67.8MHz ^{13}C NMR spectrum of Sy134/4, with 135 and 90 DEPT plots.

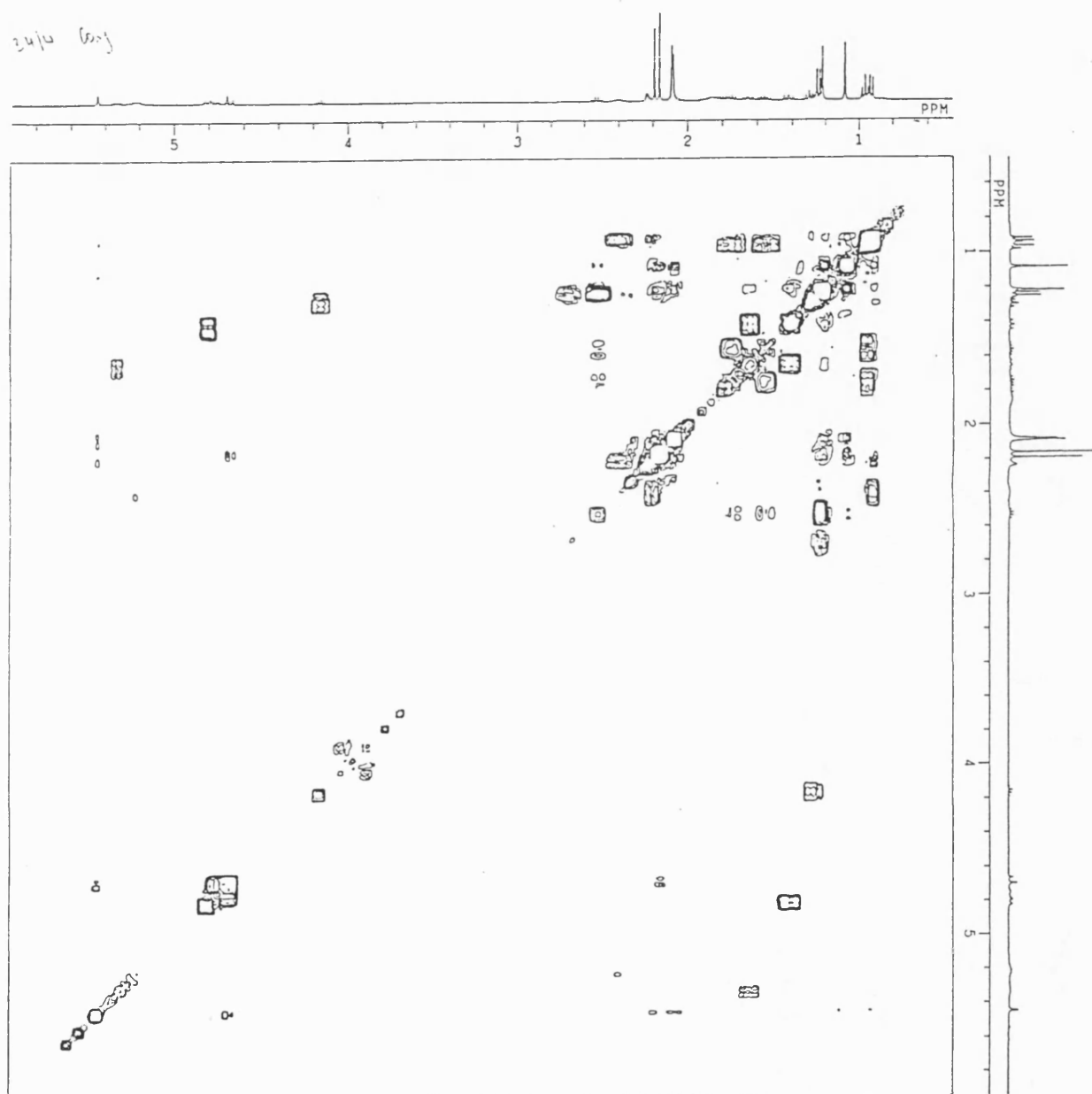


Fig 2.33 Sy134/4 COSY.

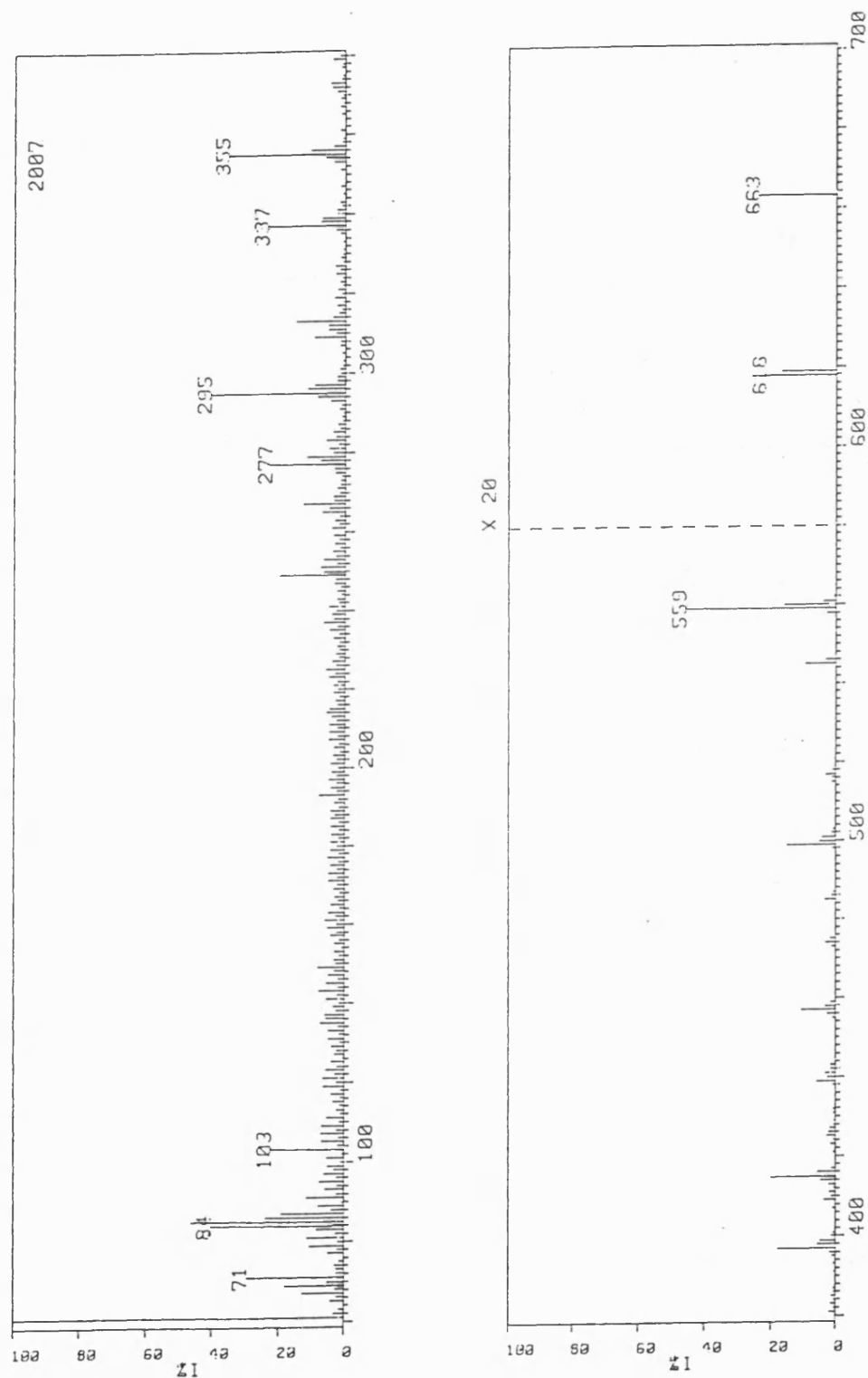
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0:377114C 8 SY134/4-ISO-BUT. C.I.
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Fig 2.34 Sy134/4 Mass spectrum.

The overall structure is similar to Sy215/1 and Sy134/6 for most of the positions. The differences occur at positions 5, 6, 7 and 17. It is likely that acylation at position 7 is the same.

In the ^{13}C NMR spectrum, the most obvious difference between this compound and Sy215/1 and its acetylated derivative is the loss of a double bond, leaving only two signals between 120ppm and 140ppm. An additional CH_3 signal at 15.8ppm is also apparent. As the differences between the compounds occur around positions 5, 6, 7 and 17, it is likely that the compound is closer to lathyrane than the other two in having a methyl group at position 17. If this is the case, then the carbon at position 6 has to be quarternary, as there is no observable coupling in the COSY between any of likely signals for the protons at positions 5, 6, 7 or 17.

If position 6 is quarternary, what are its substituents? An additional ring connection is unlikely, as the remaining portion of the molecule has already been assigned. The only realistic alternative is that the carbon at position 6 has an alcohol function as its fourth substituent. In the 135DEPT ^{13}C experiment, only three CH_2 signals are evident. However, one of these signals is of far greater intensity than the other two, and it may be possible that the two signals are coincident. Positions 1 and 20 and the CH_2 in the 2-methyl-butyrate account for three CH_2 groups. Position 5 in the ring may well be the additional CH_2 . If this is the case, then the structure of the compound has to be that in Fig 2.36.

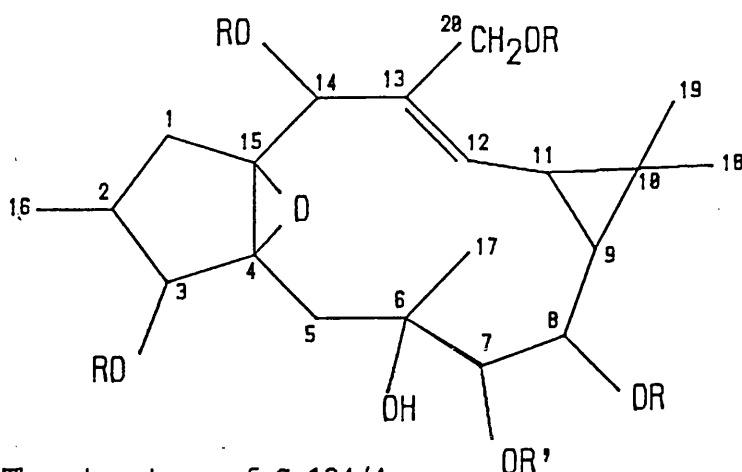


Fig 2.36: The structure of Sy134/4.

The compound would have an empirical formula $C_{33}H_{48}O_{12}$. This would give ten double bond equivalents, five accounted for by the ester carbonyls, four by the ring system and the remaining one by the double bond.

The mass spectrum seems to support this structure. A small peak at m/z 619 in the CI-MS is the largest ion, and as in Sy215/1, it is possible that this is a fragment ion, the pseudo-molecular ion $[M+1]^+$ having dehydrated from 637 with the loss of 18 units. Fragmentation is evident from both 637 and 619, with the loss of esters as free acids (see Fig 2.35).

From 637, the successive loss of acetic acid and 2-methyl butyric acid (162 mass units) gives a peak at 475. The sequential loss of three acetic acid groups give peaks at 415, 355 and 295 mass units. Alternately, the molecule can lose two acetic acid groups successively to give a peak at 517. This then loses methyl-butyrate and the remaining acetates giving peaks at 457, 397 and 295. The fragment of mass 295 then dehydrates further to 277.

From the dehydrated fragment at 619, a peak of high relative intensity (approx. 45%) is observed at 559 mass units, which suggests that this is the primary fragmentation pathway. This peak then loses either

methyl-butyrate or acetate, 102 or 60, giving peaks at 499, 457, 439, 397, 379, 337 and 277.

From each fragmentation pathway it is possible to account for the five ester groups and the alcohol, and this would support the proposed structure of the compound.

The structure is a pentaacyl derivative of 1,12-epoxy, 3-hydroxymethyl, 2,8,9,10,13-pentahydroxy, 6,6,10,14-tetramethyl tricyclo [10,3,0,0^{5,7}] pentadeca-3-ene. The acyl groups comprise four acetates and one 2-methyl butyrate. The location of the individual esters is uncertain.

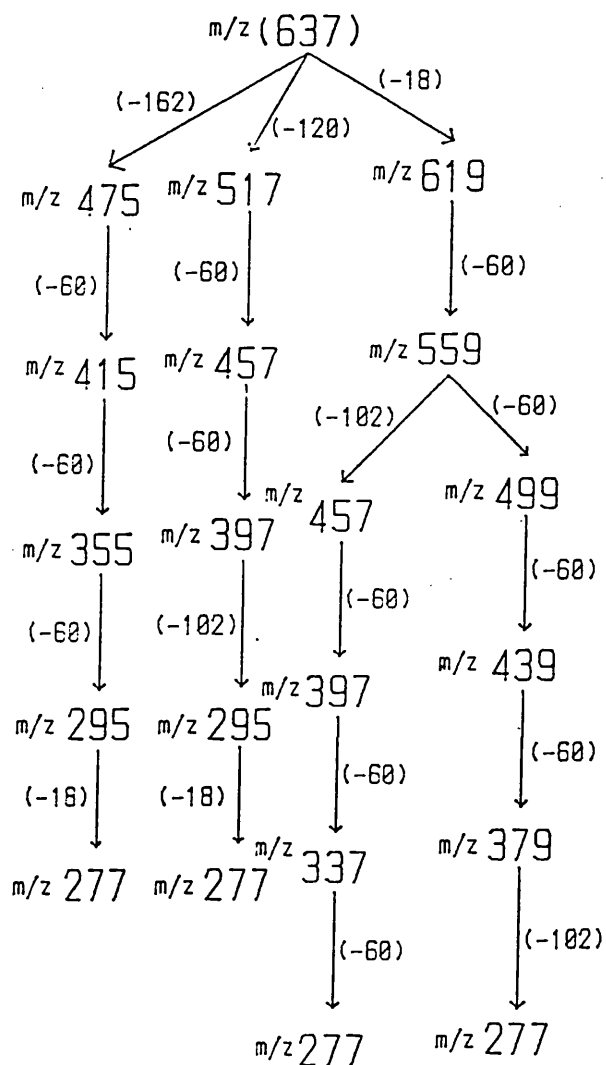


Fig 2.37: MS fragmentation pattern of Sy134/4.

Table 2.7: Sy134/4 ^1H NMR spectral assignments.

Chemical shift (ppm)	Multiplicity	Coupling constants	Integral	Assignment
0.89	d.	$^3J_{\text{H16}, \text{H2}}$ 7.5Hz	3	H16
0.93	t.	$^3J_{\text{H4}', \text{H3'}}$ 7.5Hz	3	H4'
1.05	s.		3	H19
1.18	s.		3	H18
1.21	d.	$^3J_{\text{H5}', \text{H2'}}$ 6.9Hz	3	5'
1.26	m.		1	
1.39	d.d.	$^3J_{\text{H9}, \text{H8}}$ 11.3Hz $^3J_{\text{H9}, \text{H11}}$ 9.4Hz	1	H9
1.53	m.		1	3'a
1.65	m.		1	H11
1.71	m.		1	3'b
1.82	m.		3	17
2.04	s.		3	acetate CH_3
2.05	s.		3	acetate CH_3
2.12	s.		3	acetate CH_3
2.1-2.2	m.		2	5
2.15	s.		3	acetate CH_3
2.23	m.		2	H1
2.37	m.	$^3J_{\text{H2}, \text{H16}}$ 7.5Hz	1	H2
2.49	m.	$^3J_{\text{H2}', \text{H5'}}$ 6.9Hz	1	2'
4.64	d.	$^2J_{\text{H20a}, \text{H20b}}$ 12.8Hz	1	H20a
4.72	d.	$^2J_{\text{H20b}, \text{H20a}}$ 12.8Hz	1	H20b
4.74	br.d		1	H8
5.18	br.s.		1	H7
5.19	br.d.		1	H3
5.29	d.	$^3J_{\text{H12}, \text{H11}}$ 12.4Hz	1	H12
5.40	s.		1	H14

Table 2.8: Sy134/4 ^{13}C NMR spectral assignments.

Chemical shift (ppm)	Carbon multiplicity	Assignment
170.8	q	ester carbonyl
170.7	q	ester carbonyl
170.1	q	ester carbonyl
169.7	q	ester carbonyl
129.8	CH	12
124.5	q	13
77.8	CH	4 or 15
76.6	CH	3
72.5	CH	8
71.1	CH	14
60.8	CH ₂	20
41.3	CH	2'
38.9	CH ₂	1
34.1	q	?
30.0	CH	?
29.3	CH	2
28.7	CH ₃	18
26.7	CH ₂	3', 5
26.5	CH	11
23.0	q	10
21.1	CH ₃	acetate CH ₃
20.9	CH ₃	acetate CH ₃
20.7	CH ₃	acetate CH ₃
20.5	CH ₃	acetate CH ₃
18.9	q	?
17.2	CH ₃	16
16.6	CH ₃	5'
15.8	CH ₃	17
15.2	CH ₃	19
11.5	CH ₃	4'

CHAPTER THREE.

INVESTIGATION OF *ANDROSTACHYS JOHNSONII* PRAIN.

3.1 Introduction.

Androstachys johnsonii Prain is an upright tree, 4-8m in height, although some specimens grow to 36m (Palmer, Pitman and Codd 1972). The tree is deciduous or semideciduous and in tropical areas is almost evergreen. The leaves are simple and opposite with adjacent pairs at right angles to each other. Alvin (1987) has described the detailed leaf anatomy of the species and its functional significance. The plant is unusual in having un lignified sclereids in the leaves and Alvin has suggested that these structures have an important role in water storage. The leaves vary in form but are most frequently ovate-elliptic with rounded bases and obtuse apices. Young leaves are pale green turning darker with age. The upper surface is glossy and the lower densely pubescent.

The branches and stems are grey-white to almost black. Flowers are unisexual and borne on separate plants. Male flowers occur in small spikes and the female ones singly (Palmer *et al* 1972). The fruits are typically euphorbiaceous with two to five celled capsules, three being the modal form, which dehisce at maturity.

The tree is thermophilous and is distributed from Zimbabwe and central Mozambique southwards through the eastern Transvaal, Swaziland and northern Zululand (Alvin 1987). It is gregarious, forming pure (not mixed) forest in places (Compton 1966). It grows in sandy soil, commonly along streams or on hillsides in areas of low rainfall, less than 600mm p.a.

(Alvin 1987).

Androstachys johnsonii was first described by Prain in 1905 and was a monospecific genus until 1970 when Airy Shaw described a further four species from Madagascar (Radcliffe-Smith 1987). The classification of the genus is controversial. Airy Shaw suggested a separate family, Androstachydaceae, for the genus (Willis 1973) although this has been rejected by Kew (Radcliffe-Smith 1987) and some South African botanists (van Wyk 1974). Webster (1975) places *Androstachys* in the subfamily Oldfieldioideae, tribe 15.

Androstachys johnsonii produces a timber which is dark in colour, very heavy, hard, durable and insect resistant (van Wyk 1974), hence the name ironwood. It is used for sleepers, fence posts, flooring and joinery (Palmer *et al* 1972), as well as for hut timbers (Compton 1966).

The foliage of the tree is browsed by cattle and antelopes and the fruits eaten by birds and some small game (Palmer *et al* 1972). The sawdust of the timber has a bitter taste (van Wyk 1974) and honey made from nectar of the plant is said to be poisonous (Watt and Breyer-Brandwijk 1962).

The heartwood of *Androstachys johnsonii* has been investigated by Pegel *et al* (1971) and Piacenza *et al* (1979 and 1985) who have isolated a number of oxygen containing tetracyclic diterpenoids of the beyerane class (Fig. 3.1a) and one of the atisane type (Fig. 3.1b). A similar beyerane diterpene, stachenone (Fig. 3.1c), has been isolated from the heartwood of *Spirostachys africanus* Sond. (Baarschers *et al* (1962)), a species from the same family also occurring in Swaziland.

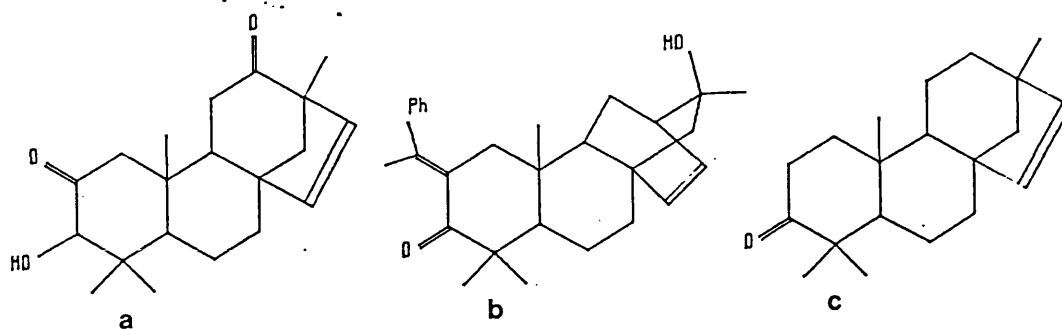


Fig 3.1 Diterpenoid compounds from *Androstachys johnsonii* and *Spirostachys africanus*. a. *ent*-3 β hydroxybeyer-15-ene-2,12-dione, b. *ent*-2-benzylidene-16 α -hydroxyatis-13-ene-3-one, c. stachenone.

3.2 Materials.

Leaves, twigs and fruits of *Androstachys johnsonii* Prain were collected in Mbuluzi Gorge, Mlawula, in the north east of Swaziland, in December 1985. The material was air dried under shade at ambient temperature and packaged in paper bags inside cardboard cartons for shipping to England. The material was stored at room temperature in the dry state at the University of Bath.

The identity of the plant material was confirmed by Mr A. Radcliffe-Smith of the Royal Botanic Gardens, Kew, Richmond, Surrey, and a voucher specimen and photographs of the species in habitat have been placed in the herbarium at Kew.

Reference samples of canophyllol and 30-hydroxy-friedelan-3-one were obtained from Dr C. Betancor, Instituto de Productos Natureles Organicos, C.S.I.C., Departamento de Quimica Organica de la Universidad de la Laguna, Tenerife.

All solvents were of Laboratory Grade standard and were redistilled from glass prior to use.

3.3 Methods.

3.3.1 Extraction.

Leaves and twigs of *Androstachys johnsonii* (1kg) were macerated with methanol (approx. 10l) at room temperature for 48 hours. The extract was decanted and the plant material extracted with a further portion of fresh methanol. The two extracts were combined and reduced under vacuum to a small volume (approx. 200ml).

3.3.2 Partitioning.

The concentrated methanol extract was partitioned between methanol-water 10:1 (500ml) and cyclohexane (500ml). Any precipitate was filtered off and retained. The two fractions were separated. The aqueous methanol layer was extracted with a further three portions of cyclohexane (100ml). The cyclohexane fraction was extracted with three portions of methanol-water 10:1 (100ml). The aqueous methanol fractions were combined, as were the cyclohexane fractions. The cyclohexane fraction was evaporated under reduced pressure to a small volume (approx. 100ml).

Equal portions of water and diethyl ether (500ml) were added to the aqueous methanol fraction. Any precipitate was filtered off and retained. The two fractions were separated and the aqueous fraction extracted with a further two portions (250ml) of diethyl ether. The ether fractions were combined and extracted with aqueous sodium carbonate 1%w/v (3x 200ml) to remove chlorophyll. The combined sodium carbonate fractions were backwashed with a further single portion of ether (100ml). The ether fractions were combined, dried over anhydrous magnesium sulphate, and evaporated under reduced pressure to dryness.

3.3.3 Chromatography.

The dried ether soluble fraction (8.5g) was fractionated on a silica gel column (Kieselgel 60, 70-230 mesh, 800g). The silica gel was slurried in *n*-hexane and the sample eluted with 1.5l of *n*-hexane ethyl acetate 80:20, followed by 200ml of a 50:50 mixture of the two solvents, followed by neat ethyl acetate.

Five fractions were collected by colour, and the solvent removed under reduced pressure. The solid material remaining from each fraction was redissolved in ethyl acetate.

3.3.4 Spectroscopic methods.

The NMR instruments used were Jeol GX400 and GX270 F.T. spectrometers. The 399.65 MHz ^1H spectrum was collected with 48 scans over a frequency width of 5000Hz and 32K data points, giving a resolution of 0.31Hz. The 67.8MHz noise-decoupled ^{13}C and DEPT spectra were collected with 9000 scans over a frequency width of 18050.5Hz and 16K data points to give a resolution of 1.1Hz/pt. The 100.4MHz ^{13}C - ^1H shift correlation spectrum was obtained with 32 scans per slice and a data matrix of 1024x512 complex points. The row and column frequencies were 8103.7Hz and 1400.0Hz respectively giving resolutions of 15.8Hz and 2.27Hz.

Mass spectrometric measurements were made on a V.G. Analytical 7070E instrument (V.G. Analytical Ltd., Manchester), housed in the School of Chemistry at the University of Bath.

3.4 Results and discussion.

3.4.1 Fractionation.

A portion of the insoluble material (0.2mg) from the partitioning of the aqueous methanol phase with cyclohexane (1.2mg) was dissolved in a 0.1M aqueous solution of potassium hydroxide (1ml). The material dissolved rapidly forming an orange solution. It was concluded that flavonoids may be present but that it was not worth pursuing due to the paucity of material.

The ether soluble fraction (8.5g) of the leaf material was a black, tarry substance. On separation on the silica gel column, five fractions were collected by colour. The first three were yellow and the last two were green. The fourth fraction, after evaporating to dryness and redissolving in ethyl acetate formed an immediate, heavy precipitate. The precipitate was filtered off and recrystallised from hot ethyl acetate. The mother liquor of the precipitate was evaporated to dryness and redissolved in hot ethyl acetate and left to recrystallise overnight. The crystals formed were colourless needles. The mother liquor was recrystallised until no further crystals were formed, and the crystals bulked for instrumental analysis. A total of 84mg of crystalline material was collected. The crystals were given the code number An10. Samples were prepared for Mass spectrometry, N.M.R. spectroscopy and single crystal X-ray studies.

3.5 Structure elucidation of An10.

The mass spectrum of An10 is presented in Fig.3.2 and shows a small molecular ion peak at 442. This could give an empirical formula of $C_{30}H_{50}O_2$.

The Eight Peak Index of Mass Spectra (1974) refers to a compound, canophyllol with a molecular ion peak at m/z 442 (2.59%) and fragmentation peaks at m/z 411 (84%), m/z 273 (100%), m/z 247 (35%), m/z 137 (58%), m/z 109 (36%), m/z 95 (35%), m/z 81 (32%), and m/z 69 (32%). This compound (Fig.3.3) was isolated from *Calophyllum inophyllum* L. and reported by Govindachari *et al* (1967).

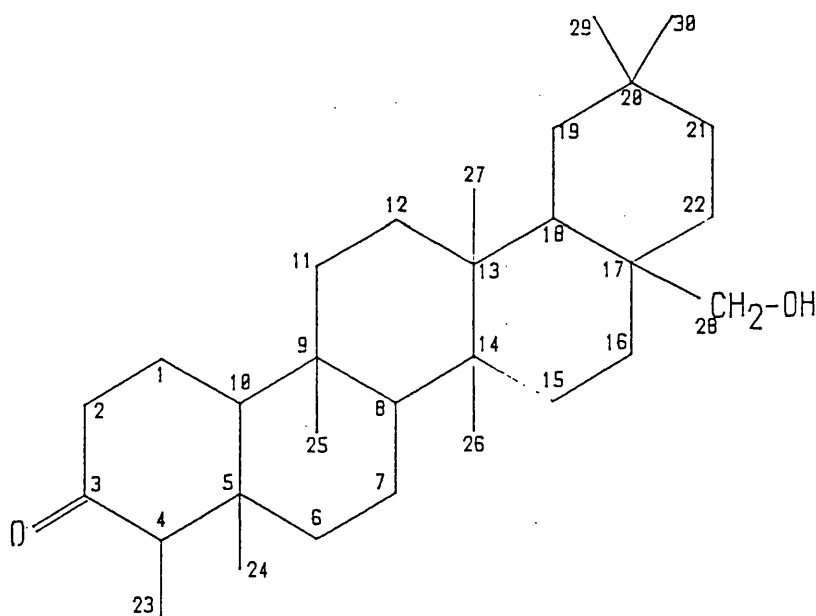


Fig3.3 Canophyllol, 28-hydroxyfriedelan-3-one from *Calophyllum inophyllum*.

The fragmentation pattern is the same as that of the compound from *Androstachys johnsonii* and a tentative identification was made on this evidence. The CH_2-OH group was assigned to position 17 on account of the the loss of a 31 molecular mass fragment from m/z 442 to m/z 411 suggesting bridgehead substitution for this group.

The ^1H and ^{13}C one-dimensional NMR spectra, and the two-dimensional C-H shift correlation are presented as figures 3.4 to 3.6.

Overall, the NMR spectra for the compound are consistent with the above structure. The strong singlet at 3.6ppm in the proton spectrum integrates to two protons and is consistent with a methylene group adjacent to an alcohol function. The presence of a single methyl doublet and six methyl singlets in the proton spectrum suggest that the compound may be of the friedeline type, which commonly have either a ketonic function at carbon atom 3 or, in the epi-friedeline variation, an alcohol in this position. The ^{13}C DEPT experiments confirm the presence of seven methyl groups and indicate twelve CH_2 groups, four CH groups and seven quaternary carbon atoms, one of which lies in the carbonyl region (213.2ppm).

Patra *et al* (1987) have compared the ^{13}C NMR data of thirty two derivatives of friedelane and have made the assignments using experiments to determine the number of protons attached to each carbon atom, from chemical shift changes on changes of functionality using chemical shift theory and by comparison between the compounds. Canophyllol was one of the derivatives used in the study and the ^{13}C data is in exact agreement with that of An10.

A ^{13}C - ^1H heteronuclear shift correlation experiment (Fig.3.5) was performed which enabled assignments in the ^1H spectrum to be made on the basis of the assignments of Patra *et al* in the ^{13}C spectrum. The NMR data for the compound isolated by Govindachari *et al*, although not detailed, was in agreement with that of An10.

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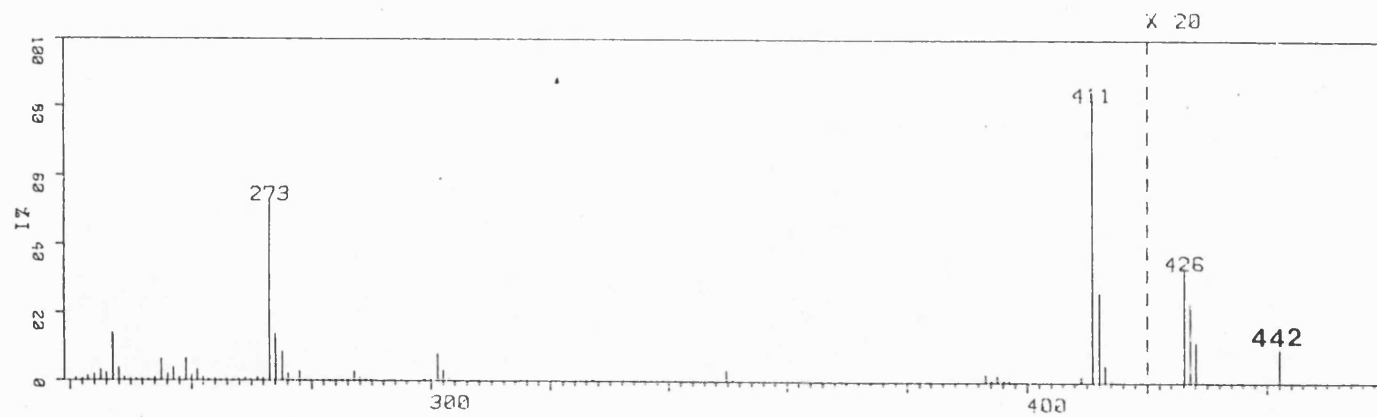
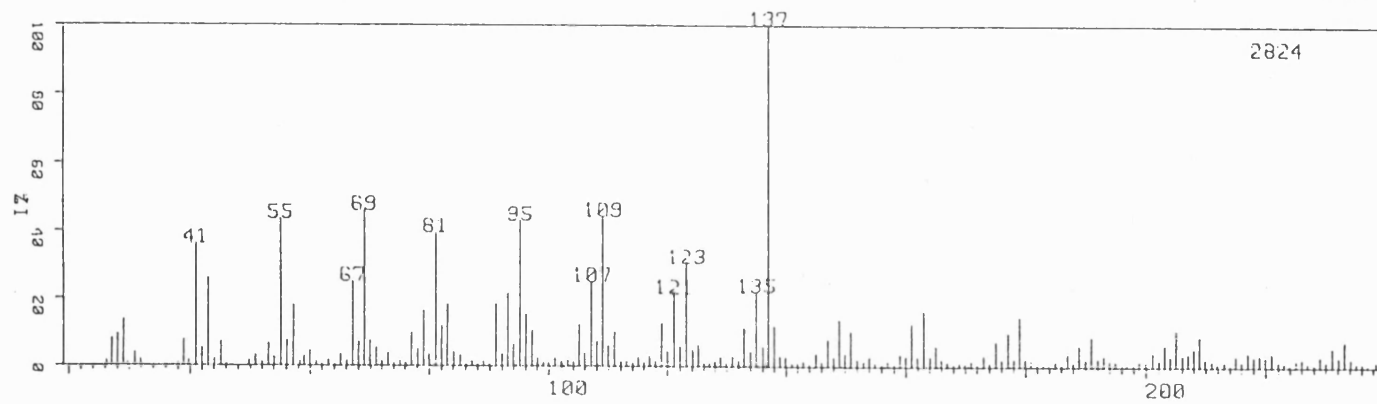


Fig 3.2 An10 Mass spectrum.

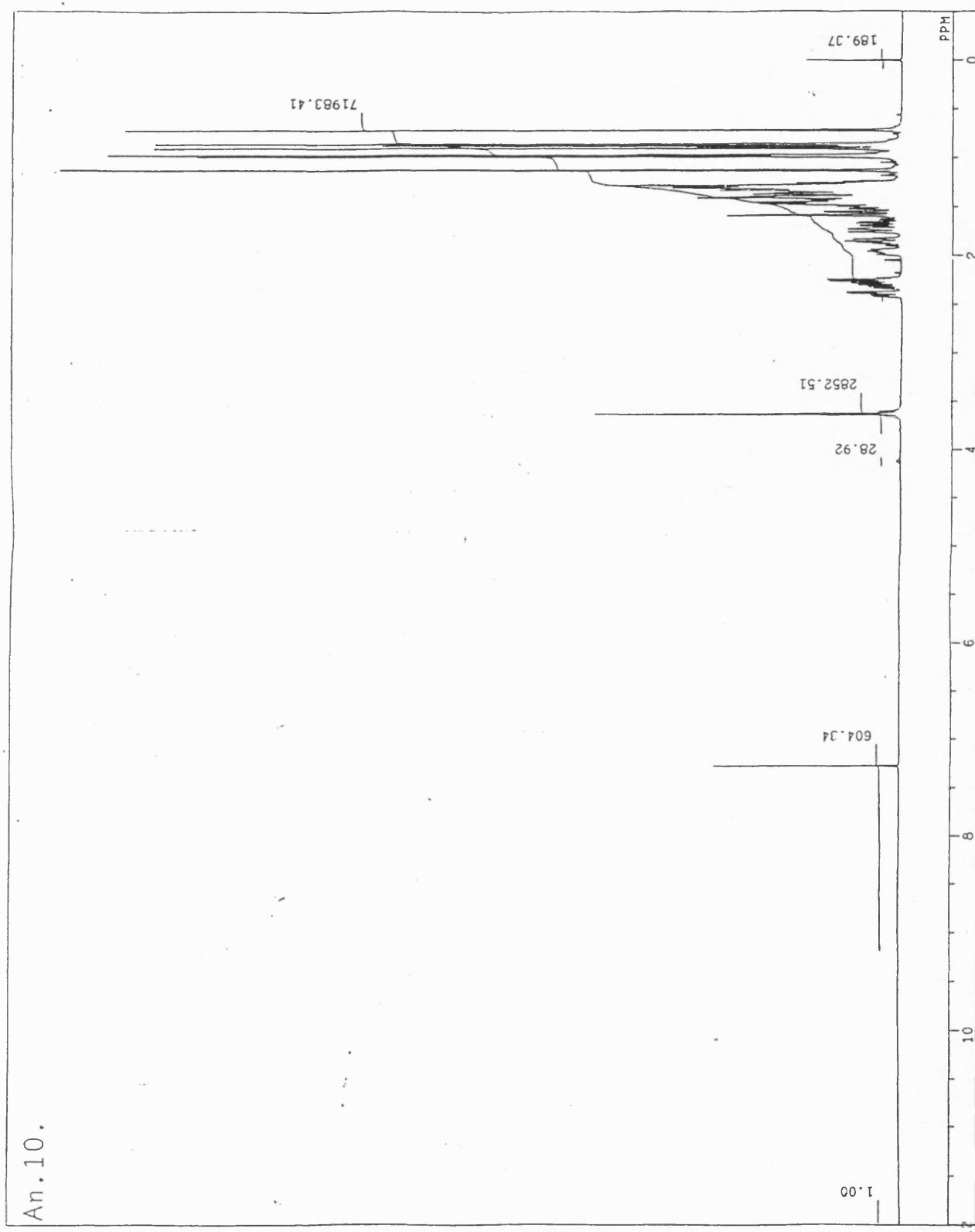


Fig 3.4a 400MHz ^1H NMR spectrum of An10.

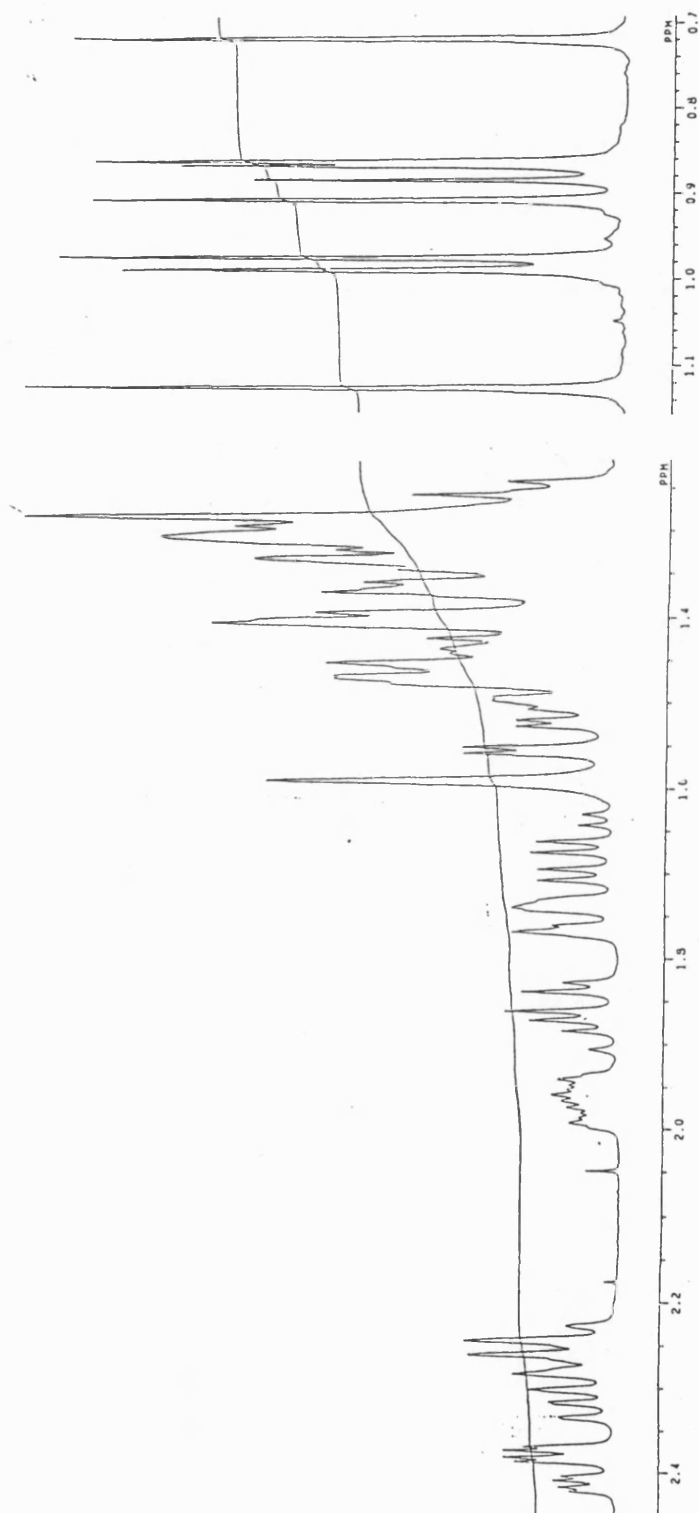


Fig 3.4b ^1H NMR spectrum of An10 expansion.

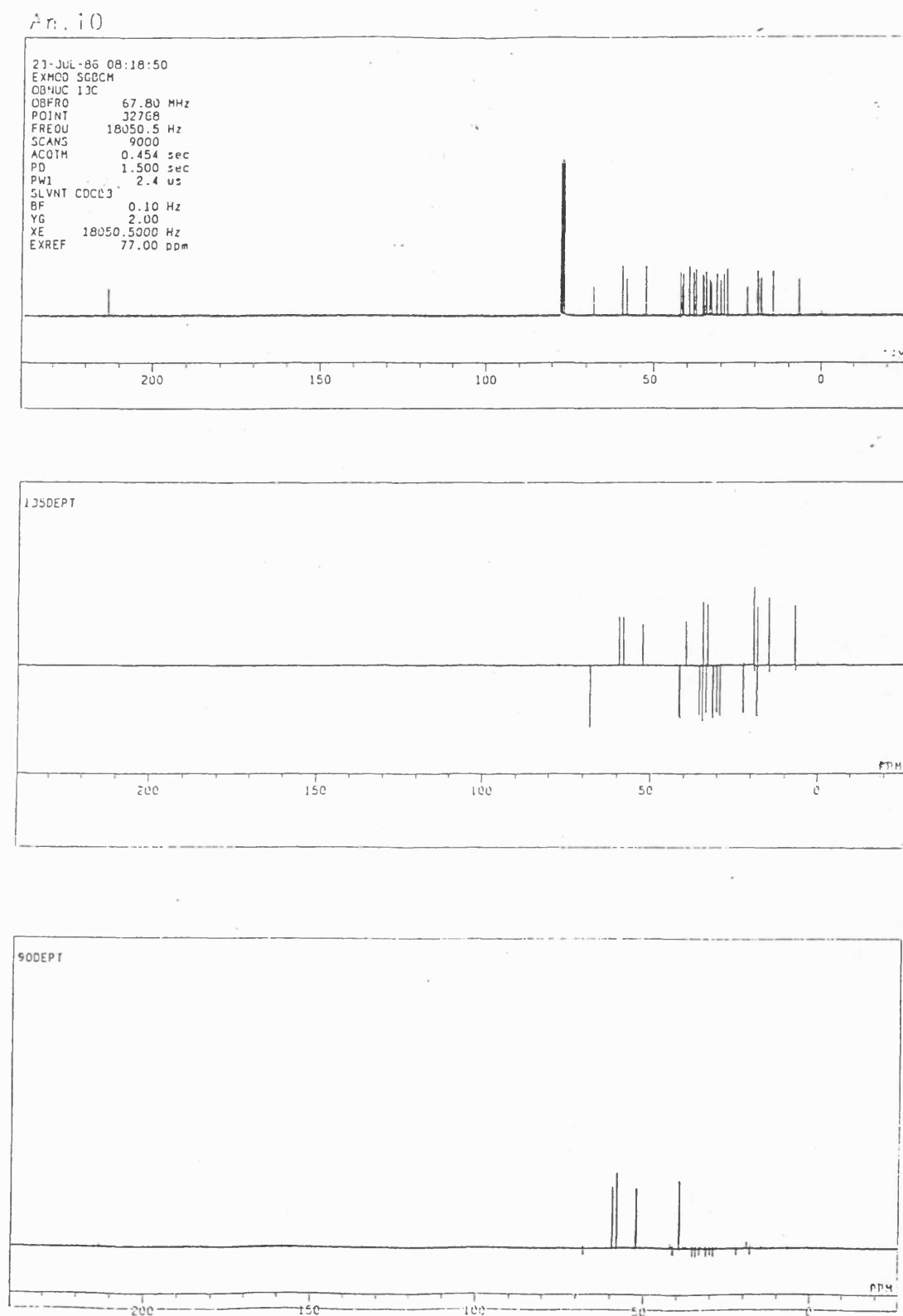


Fig 3.5 67.8MHz ^{13}C NMR spectrum of An10, with 135 and 90 DEPT plots.

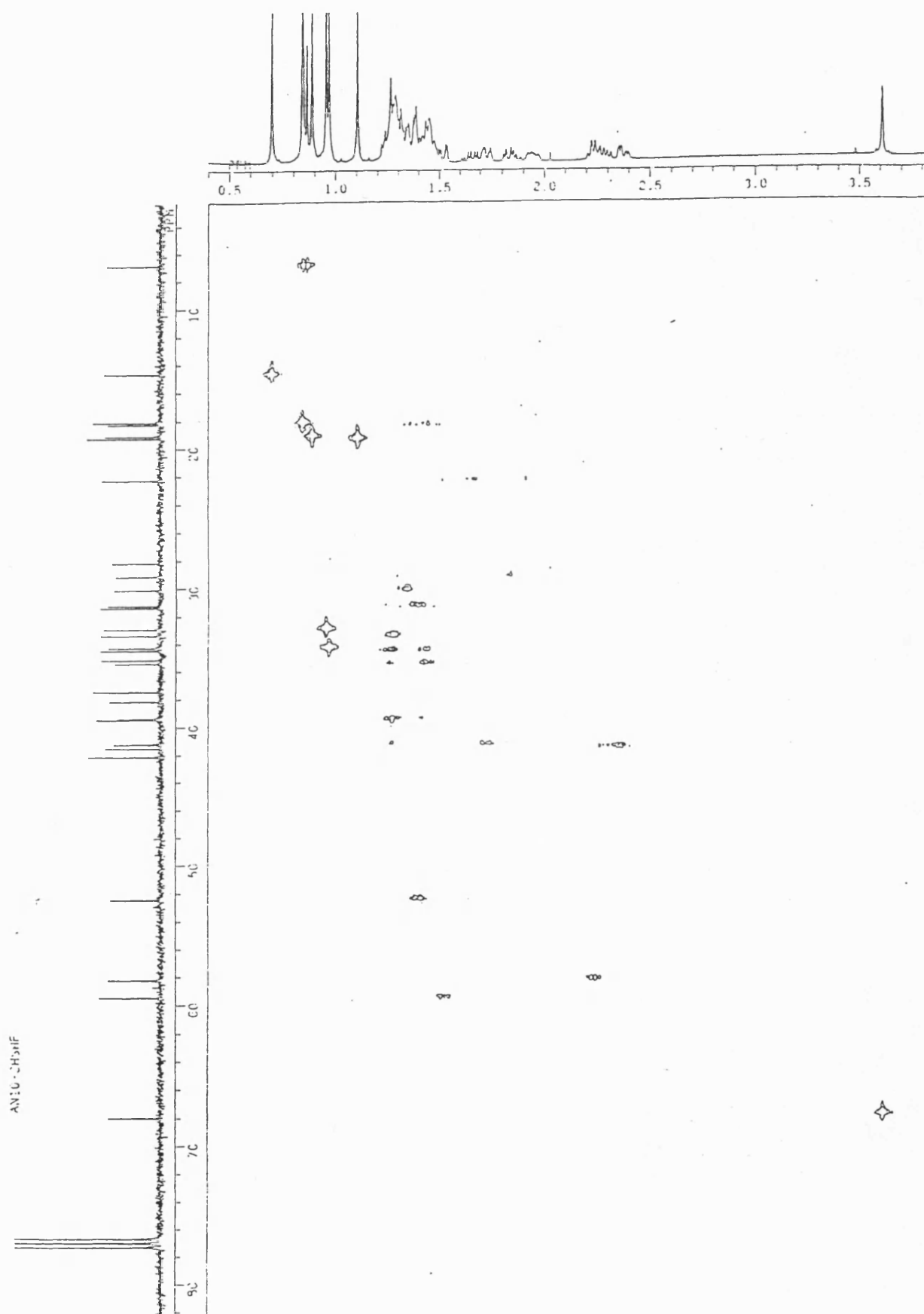


Fig 3.6 An10 ^{13}C - ^1H shift correlation.

The assignment of the methyl signals is straightforward. The doublet with coupling constant of 6.4Hz at 0.88ppm corresponds to the methyl group at position 23 adjacent to a single proton at position 4. The six other methyl signals are sharp singlets, indicating quaternary substitution of the adjacent carbon atoms and can be assigned from the ^{13}C - ^1H shift correlation. In the ^1H spectrum, the unresolved region between 1.24ppm and 1.51ppm contains signals for eighteen protons from positions 6(1H), 7, 8, 11, 12, 15, 16(1H), 18, 19, 21 and 22.

In the region 1.51ppm - 2.4ppm, signals due to a further eight protons can be seen. As expected, the signals due to protons on positions 1, 2, 4 and 10 occur in this region due to the deshielding effect of the carbonyl group at position 3. One proton from position 6 and one from position 16 also resonate in this region.

The proton attached to position 10 on the skeleton resonates at 1.54ppm. The splitting pattern is one of a doublet of doublets with vicinal couplings of 12.9Hz to the axial proton and 2.9Hz to the equatorial proton at position 1, indicating that H_{10} is an axial proton.

The protons at position 1 are magnetically non-equivalent. The axial proton shows a quartet of doublets 1.68ppm. The larger coupling constant of 12.9Hz is due to splitting by the geminal proton, the axial proton at position 2 and H_{10} . The second coupling constant of 5.0Hz is due to splitting by the equatorial proton at position 2 which resonates at 2.40ppm.

The equatorial proton at position 1 resonates at 1.97ppm. The signal is an incompletely resolved doublet of doublets of triplets with a geminal

coupling constant of 12.9Hz, a vicinal coupling constant of 2.9Hz due to splitting by H₁₀, a vicinal coupling constant of 7.2Hz due to the axial proton at position 2 and a vicinal coupling constant of 2.2Hz due to the equatorial proton at position 2.

The protons at position 2 are adjacent to the carbonyl carbon and are the most deshielded of the ring protons. The doublet of doublets of doublets at 2.40ppm for the equatorial proton shows three coupling constants. A geminal coupling constant of 14.3Hz and two vicinal coupling constants, one of 5.0Hz for the H₂-equatorial-H₁-axial splitting, and one of 2.2Hz for the H₂-equatorial-H₁-equatorial splitting. The axial proton triplet of doublets at 2.29ppm overlaps with that of the proton at position 4, but the 14.3Hz geminal coupling and H₁ axial vicinal coupling 7.2 Hz can still be measured.

The single proton at position 4 is coupled to the methyl group at position 23. The expected quartet is overlapped by the signal for the axial proton at position 2, but the coupling constant of 6.4Hz, in agreement with that of the methyl doublet at 0.88ppm, can be measured.

A broadened doublet resonating at 1.75ppm can be assigned to one of the protons at position 6, having a geminal coupling constant of about 11.5Hz.

One of the protons at position 16, deshielded by its proximity to the alcohol group at position 28, resonates as a multiplet at 1.85ppm. Two coupling constants can be measured, a geminal constant of 13.4Hz and an unidentified vicinal coupling of 4.3Hz, possibly to H₁₅.

Table 3.1: An10 ^1H NMR data.

Chemical shift (ppm)	Multiplicity	Coupling constants	Integral	Assignment
0.72	s.		3	24
0.87	s.		3	25
0.88	d.	$^3J_{\text{H}23, \text{H}4} 6.4\text{Hz}$	3	23
0.91	s.		3	26
0.98	s.		3	29
0.99	s.		3	30
1.12	s.		3	27
1.24-1.51		unresolved	18	6 (1xH), 7 (2xH), 8 (1xH), 11 (2xH), 12 (2xH), 15 (2xH), 16 (1xH), 18 (1xH), 19 (2xH), 21 (2xH), 22 (2xH).
1.54	d.d.	$^3J_{\text{H}10, \text{H}1a} 12.9\text{Hz}$ $^3J_{\text{H}10, \text{H}1e} 2.9\text{Hz}$	1	10
1.68	q.d.	$^2J_{\text{H}1a, \text{H}1e} 12.9\text{Hz}$ $^3J_{\text{H}1a, \text{H}10} 12.9\text{Hz}$ $^3J_{\text{H}1a, \text{H}2a} 12.9\text{Hz}$ $^3J_{\text{H}1a, \text{H}2e} 5.0\text{Hz}$	1	1a
1.75	br.d.	$^2J_{\text{H}6} 11.5\text{Hz}$	1	6
1.85	m.	$^2J_{\text{H}16} 13.4\text{Hz}$	1	16
1.97	d.d.t.	$^3J_{\text{H}16, \text{H}15} 4.3\text{Hz}$ $^2J_{\text{H}1e, \text{H}1a} 12.9\text{Hz}$ $^3J_{\text{H}1e, \text{H}2a} 7.2\text{Hz}$ $^3J_{\text{H}1e, \text{H}10} 2.9\text{Hz}$ $^3J_{\text{H}1e, \text{H}2e} 2.2\text{Hz}$	1	1e
2.28	q.	$^3J_{\text{H}4, \text{H}23} 6.4\text{Hz}$	1	4
2.29	t.d.	$^2J_{\text{H}2a, \text{H}2e} 14.3\text{Hz}$ $^3J_{\text{H}2a, \text{H}1a} 12.9\text{Hz}$ $^3J_{\text{H}2a, \text{H}1e} 7.2\text{Hz}$	1	2a
2.40	d.d.d.	$^2J_{\text{H}2e, \text{H}2a} 14.3\text{Hz}$ $^3J_{\text{H}2e, \text{H}1a} 5.0\text{Hz}$ $^3J_{\text{H}2e, \text{H}1e} 2.2\text{Hz}$	1	2e
3.60	s.		2	28

Table 3.2: An10 ^{13}C NMR data.

Chemical shift (ppm)	Carbon multiplicity	Assignment (Patra <i>et al</i> 1987)
6.8	CH ₃	23
14.6	CH ₃	24
18.0	CH ₂	7
18.2	CH ₃	25
19.0	CH ₃	26
19.2	CH ₃	27
22.2	CH ₂	1
28.1	q	20
29.1	CH ₂	16
30.0	CH ₂	12
31.2	CH ₂	15*
31.3	CH ₂	21*
32.8	CH ₃	29
33.3	CH ₂	22
34.2	CH ₃	30
34.4	CH ₂	19
35.1	q	17
35.4	CH ₂	11
37.4	q	9
38.1	q	14
39.3	q	13
39.4	CH	18
41.2	CH ₂	6
41.5	CH ₂	2
42.1	q	5
52.4	CH	8
58.2	CH	4
59.4	CH	10
68.0	CH ₂	28
213.2	q	3

*The assignments for carbon atoms 15 and 21 may be reversed.

A single crystal X-ray analysis confirmed the structure and established the stereochemistry of An10. The structure was solved by direct methods and is presented in Fig.3.7 showing the stereochemistry at each position.

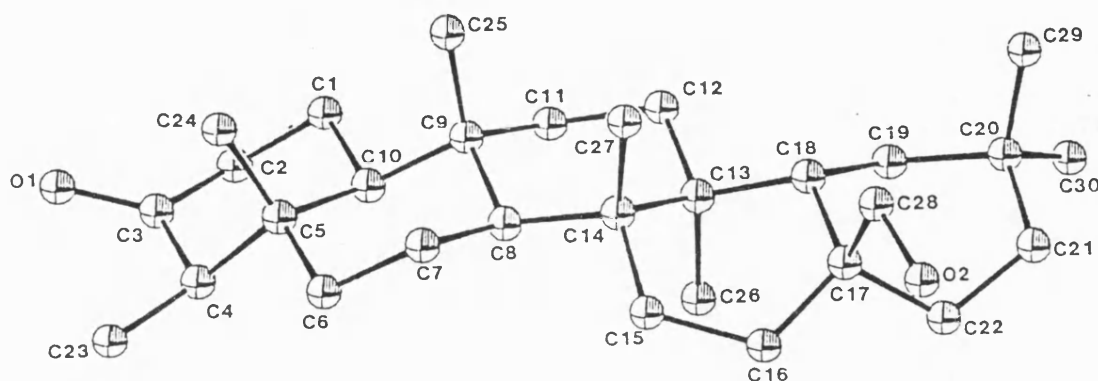


Fig.3.7 The ORTEP diagram for An10, 28-hydroxyfriedelan-3-one.

Friedelane (also known as friedooleanane) and its derivatives are fairly common triterpenes found in a number of plant families. Risk and El-Missiry (1986) have reviewed the distribution of triterpenoids within the Euphorbiaceae. Friedeline and friedeline derivatives have been reported from thirteen species in eight genera, *Antidesma*, *Apurosa*, *Bischofia*, *Bridelia*, *Euphorbia*, *Glochidion*, *Phyllanthus*, *Putranjiva* and *Sapium*.

The compound isolated from *Androstachys johnsonii*, 28-hydroxyfriedelan-3-one, is a less common derivative of friedelane and has been reported previously from the following genera: *Amanoa* (Fang et al 1985), *Antidesma* (Rizvi et al 1980), *Calophyllum* (Govindachari et al 1967), *Castanopsis* (Hui et al 1976), *Elaeodendron* (Anjaneyulu et al 1980), *Euonymus* (Kumar et al 1985), *Hydnocarpus* (Gunasekera et al 1977), *Lithocarpus* (Hui et al 1975), *Maytensus* (Nozaki et al 1986) and *Quercus* (Kulshreshtha, D.I.C. et al 1971),

The compound isolated by Gunasekera *et al* (1979) was initially thought to be 29-hydroxyfriedelan-3-one and was given the trivial name octandronol. This structure was contested by Betancor *et al* (1980) who isolated both the 29- and the 30-hydroxy compounds from *Catha cassinoides*. They have suggested that the structure for octandronol must be the same as for canophyllol, as the physical and spectral data for octandronol and 29-hydroxyfriedelan-3-one were different.

Authentic samples of canophyllol and the 30-hydroxy derivative from *Catha cassinoides* were obtained from Dr Betancor. The ^1H spectrum of canophyllol was found to be identical with that of the compound from *Androstachys*. The ^1H spectrum for 30-hydroxy-friedelan-3-one differed from that of the *Androstachys* compound in a number of respects, most noticeably the signal for the methylene group adjacent to the alcohol group. The signal in the 30-hydroxy compound resonates at 3.27ppm, and whereas the methylene signal of the 28-hydroxy derivative is a sharp singlet, this signal is a multiplet, suggesting hindered rotation about the axis.

PART TWO.

AN EVALUATION OF COUNTERCURRENT CHROMATOGRAPHY IN THE FRACTIONATION OF
NATURAL PRODUCTS.

CHAPTER FOUR.

INTRODUCTION.

4.1 COUNTERCURRENT DISTRIBUTION.

4.1.1 Background.

When a solute is dissolved in a mixture of two immiscible liquids, at equilibrium it is distributed between the liquids according to its partition coefficient. This can be given the symbol K , and is defined as the ratio of the concentration of the solute in the one phase to the other.

$$K = \frac{[\text{concentration of solute in phase 1}]}{[\text{concentration of solute in phase 2}]} \quad \dots 1$$

There is no convention about which phase is the numerator and which the denominator, but in countercurrent distributions and chromatography the mobile phase is commonly held as numerator.

Partitioning of solutes between two immiscible liquids is frequently used in isolation procedures as a means of producing a solution enriched with the solutes of interest and from which unwanted solutes have been removed. In the extreme case, the solute of interest is entirely separated from unwanted material and the purification process is achieved in one step. Such a purification is the exception rather than the rule and single stage liquid-liquid partitioning more commonly yields an enriched solution of the solutes of interest together with such other solutes which have

similar partitioning characteristics in the liquid phases chosen.

A single stage liquid-liquid partitioning procedure can be achieved using a laboratory separatory funnel. Consider the situation in which a solute is dissolved in a liquid and placed in a separatory funnel. If an equal volume of a second, immiscible liquid is added then the solute will partition between the two liquids according to its partition coefficient. If the solute in question has a partition coefficient $K=4$ in the particular system then the fraction of solute in phase 1 can be calculated from the equation

$$p = \frac{K}{1+K} \quad \dots 2$$

For convenience we shall nominate phase 1 as the upper phase, and phase 2 as the lower phase. The fraction of solute in phase 2, q , will be the difference between unity and the fraction in phase 1.

$$q = 1-p \quad \dots 3$$

Statistically, p is the probability of a molecule of solute being found in the upper phase, and q the probability of the molecule being found in the lower phase.

The sum of p and q will always be unity. In the chosen example, it can be shown that at equilibrium, 80% of the solute will be present in phase 1, and 20% in phase 2.

If a series of separatory funnels is set up with equal portions of the lower phase from the above example in each funnel and the same solute partitioned between equal volumes of upper and lower phase in the first

funnel, at equilibrium the upper phase would contain 80% of the solute and the lower phase 20%. The upper phase is then removed and placed in the second funnel and a fresh portion of upper phase is placed in funnel one. The funnels are shaken and allowed to equilibrate. By the same calculation it can be seen that funnel one will have 4% of the solute in the lower phase and 16% in the upper. Funnel two will have 16% of the solute in the lower phase and 64% in the upper. If the process is repeated, after six transfers funnel one will contain 0.03%, funnel two 0.64%, funnel three 5.12%, funnel four 20.48%, funnel five 40.96% and funnel six 32.77% of the starting material. If there were one hundred funnels in the series it is possible to envisage from the above example that the solute would be concentrated in a limited number of funnels, and the funnels in which the solute will be found can be predicted from the knowledge of the partition coefficient of the particular solute.

If a second solute with a partition coefficient $K=0.25$ was included with the first solute at the beginning of the experiment, it can be appreciated that the first solute will be carried through the series of funnels at a faster rate than the second solute and provided that a sufficient number of funnels were available, the two solutes would eventually be separated. This procedure is analogous to a countercurrent distribution.

4.1.2 Binomial distribution.

Martin and Synge (1941) recognised that the countercurrent distribution can be described in terms of a binomial expansion

$$(q+p)^n = 1 \qquad \dots 4$$

which describes the probability of an event occurring at each trial in a

series of n trials, where p and q are the probabilities of the event occurring or not occurring in a single trial. As has been shown, p and q are related to the partition coefficient of the solute in question.

$$p = \frac{\text{amount of solute in upper phase}}{\text{total solute}}$$

$$p = \frac{C_1 V_1}{C_1 V_1 + C_2 V_2} \quad \dots 5$$

where C_1 and C_2 are the concentrations of solute in phases 1 and 2 respectively, and V_1 and V_2 are the volumes of phases 1 and 2 respectively.

Similarly,

$$q = \frac{\text{amount of solute in lower phase}}{\text{total solute}}$$

$$q = \frac{C_2 V_2}{C_1 V_1 + C_2 V_2} \quad \dots 6$$

$$\frac{p}{q} = KR \quad \dots 7$$

where K is the partition coefficient and R is the ratio of volumes of the two phases.

Rearranging equations 7 and 3 and solving for p gives

$$\begin{aligned} p &= KRq \\ &= KR(1-p) \\ &= KR - KRp \\ p + KRp &= KR \\ p(1 + KR) &= KR \\ p &= \frac{KR}{1 + KR} \quad \dots 8 \end{aligned}$$

Similarly, it can be shown that

$$q = \frac{1}{KR + 1} \quad \dots 9$$

Substituting equations 8 and 9 into equation 4 gives

$$\left[\frac{1}{KR + 1} + \frac{KR}{1 + KR} \right]^n = 1 \quad \dots 10$$

where n is the number of transfers of the mobile phase that have been made. For low values of n , it is possible to calculate the fraction of solute in each container using this equation.

For large values of n , the fraction of solute to be found in any specified container can be calculated from probability statistics. The probability of an event occurring, in our instance finding molecules of solute in a particular container, is determined by the partition coefficient of the solute in the particular solvent system. If the probability of an event occurring is p , and q is the probability of the event not occurring, ($q = 1 - p$), then the probability of the event occurring on a particular trial can be calculated from the following equation, where $f_{n,r}$ is the probability of the event occurring on trial r , after a total of n tries.

$$f_{n,r} = \left[\frac{n!}{r!(n-r)!} \right] p^r q^{(n-r)} \quad \dots 11$$

(Hall and Knight 1946)

In the case of counter current distribution, n is the number of transfers and r is the specific tube of interest.

Substituting for p and q from equations 8 and 9 gives

$$\begin{aligned} f_{n,r} &= \left[\frac{n!}{r!(n-r)!} \right] \left[\frac{KR}{1+KR} \right]^r \left[\frac{1}{1+KR} \right]^{n-r} \\ &= \left[\frac{n!}{r!(n-r)!} \right] \left[\frac{(KR)^r}{(1+KR)^r} \right] \left[\frac{1}{(1+KR)^{n-r}} \right] \\ &= \left[\frac{n!}{r!(n-r)!} \right] (KR)^r \left[\frac{1}{1+(KR)} \right]^n \quad \dots 12 \end{aligned}$$

The mean of the binomial expansion can be calculated from

$$r_{\max} = np.$$

In the counter current distribution, the mean describes the tube which contains the maximum concentration of solute.

Substituting for p gives

$$r_{\max} = n \left[\frac{KR}{1 + KR} \right]$$

The standard deviation,

$$s = \sqrt{npq} \quad ,$$

gives the distance from the mean (r_{\max}) to the inflection of the curve. Ninety five percent of the solute will be contained within the tubes which lie at twice the standard deviation on either side of the tube containing the maximum solute.

Martin *et al*(1969) have published a set of programs in Fortran for performing a countercurrent distribution simulation, in which they create an array in which each point is regarded as an exchange in the counter current distribution and use the value at a particular point to calculate the value for the following point, using the value for p in equation 2 as the basis for the calculation.

4.2 Counter current chromatography.

4.2.1 General.

Counter current distribution processes as carried out in Craig or O'Keefe apparatus can be regarded as discontinuous processes in which the system is allowed to equilibrate between transfers of mobile phase from one stage to the next. They are not without their drawbacks, however, and a number of alternative approaches to achieving liquid-liquid partition systems have been developed.

The Craig apparatus is probably the best known of the counter current distribution apparatus and was more frequently encountered in the 1960's and '70's than today. A succinct description of a counter current apparatus is given by Sixma and Wynberg (1964). Mandava *et al* (1982a) give the following disadvantages of counter current distribution which have led to the decline in the use of the technique:

- i. The equipment is complex.
- ii. The system sometimes leads to the formation of emulsions which may be difficult to break.
- iii. The volume of solvent required is fixed by the size of the apparatus and can be large.
- iv. The time taken for effective separation of compounds can be very long.

Other commonly quoted disadvantages are that the equipment requires skilled operators and that, being made of glass with ground glass connections, once a piece is broken exact replacement is difficult,

leading to problems of leakage at the replacement joint. Cleaning the apparatus is laborious and time consuming.

Counter current chromatography differs from counter current distribution in that the process is continuous and equilibrium of solute between the two immiscible phases is never achieved. In other respects, however, the two are very similar. Both rely on differential partitioning of solutes between two immiscible phases for separation. Solid supports, as are used in conventional liquid chromatography are avoided and problems of irreversible adsorption are not encountered. Sample recovery is therefore good (Ito 1984). Other problems with solid support chromatography, for example, chemical reactions such as acyl shift in diterpene esters (Hecker 1971, Upadhyay *et al* 1976) on silica gel, are avoided.

A number of different forms of counter current chromatography have been developed. Dr Yoichiro Ito has been responsible for many of these developments and is the author or co-author of many of the publications on the techniques. He has classified counter current chromatography into two basic types, hydrostatic equilibrium systems and hydrodynamic equilibrium systems depending on the overall movement of the apparatus and thus the stationary phase with respect to the mobile phase. In hydrostatic equilibrium systems, the apparatus is stationary, i.e. the coil or columns of the apparatus do not move and the mobile phase moves only under the influence of gravity, whereas in the hydrodynamic equilibrium systems the coil or columns of the apparatus move, normally by way of rotation in a centrifuge or simply about their own axes. The differentiation used by Ito is based on the engineering of the apparatus and not on the principles of the separation mechanism.

A number of reviews on counter current chromatography in general have been published. Mandava, Ito and Conway (1982a) have discussed the historical development and early instrumentation of counter current chromatography with a brief overview of counter current distribution techniques. Ito and Bowman (1971) reviewed the counter current chromatography techniques available at the time and compared them with counter current distribution systems and conventional solid support chromatography. Conway (1984) has discussed recent applications of counter current chromatography and Ito (1986a) has published a paper on the recent trends in counter current chromatography. The Journal of Liquid Chromatography have devoted three entire issues to countercurrent chromatography, namely Volume 7, No. 2 (1984), Volume 8, No. 12 (1985) and Volume 11, No. 1 (1988).

Countercurrent chromatography developed naturally from countercurrent distribution. Signer (1952) reported the development of an apparatus for separating mixtures of compounds which has led to the development of locular rotatory countercurrent chromatography. Ito *et al* (1966) reported that immiscible liquids in coiled, narrow bore tubes could be segmented and made to undergo countercurrent flow and were able to demonstrate the separation of a number of dinitrophenyl derivatives of amino acids and that the separation was according to the differences in partition coefficient. From this developed counter current chromatography using helically wound tubing which has led to the development of a number of related counter current chromatography apparatus, some of which are now available commercially. Droplet counter current chromatography was first reported in 1970 by Tanimura in conjunction with Ito and other colleagues (Tanimura *et al* 1970).

A more recent apparatus for counter current chromatography is that developed by Nishizawa and Abe (1986). The device was constructed by inserting a sealed glass tube into an outer tube with a gap of one millimeter between the two. The gap between the tubes was filled with a liquid, in the published method 1-butanol which had previously been saturated with water, and a second immiscible liquid, in the example water, introduced at the top of the apparatus and allowed to flow down the tube under the influence of gravity. The inner tube was rotated on its axis to provide mixing of the two solvents and partitioning of the solutes injected into the apparatus. The separation of a number of glucosides was described. Optimum resolution was obtained at 550 r.p.m.

4.2.2 Droplet counter current chromatography (DCCC).

Droplet counter current chromatography was first described in 1970 (Tanimura *et al* 1970). The concept is extremely elegant, the apparatus simply comprising a series of glass tubes linked with PTFE micro tubing, which are filled with stationary phase and through which a second, immiscible liquid is pumped in droplets. Solutes are partitioned between droplets of the mobile phase and the stationary phase. Those with higher partitioning into the mobile phase are carried through the columns at a faster rate than solutes with lower partitioning. Turbulence within the droplets as they pass through the stationary phase is sufficient to promote adequate partitioning of the solutes between the stationary and mobile phases. The original paper described the separation of a series of dinitrophenyl amino acid derivatives in milligram quantities with a resolution of 900 theoretical plates.

The use of droplet counter current chromatography has been extensively reviewed by Hostettmann and co-workers (Hostettmann *et al* 1979,

Hostettmann 1980a, 1980b, 1981, 1983, Hostettmann *et al* 1984a, 1984b), Tanimura *et al* 1975, Orth 1981, Becker 1984, Knapp 1985, Hanke and Kubo 1987 and Kubo *et al* 1988).

Until 1980, the majority of solvent systems in use employed chloroform, methanol and water as the main components in varying proportions with additional modifiers such as propanol or butanol in a few cases, or dilute hydrochloric or acetic acid. Droplet counter current chromatography up to this stage was applied most commonly to the separation of glycosides which exhibit good partitioning between aqueous and organic phases.

Becker *et al* (1982) published the use of a non-aqueous solvent system for DCCC. They demonstrated the separation of the terpenoid components of the volatile oil from *Matricaria chamomilla* using the solvent system *n*-hexane-ethyl acetate-nitromethane-methanol (18:3:9:3), with the upper phase as the mobile phase. The introduction of non-aqueous solvent systems is significant in that it allowed the separation of relatively non-polar components. Non aqueous systems also have the advantage of easy removal of solvent by evaporation. Becker (1984) has published a discussion of the use of non-aqueous systems for DCCC. He reported a variation on his previous system for the fractionation of a mixture of valepotriates using *n*-hexane-ethyl acetate-nitromethane-methanol (8:2:2:3) again using the upper phase as mobile phase. He also reported the use of the solvent system *n*-heptane-1,2 dichloroethane-methanol (47:6:47) for the partial separation of hederagenin and oleanic acid from *Hedera helix* using the lower phase as mobile phase. The system *n*-heptane-dichloromethane-acetonitrile (50:15:35) was tested for the separation of betulenic acid, betulin, α -amyrin and cholesterol, using the lower phase as mobile phase. Cholesterol and α -amyrin could not be separated with this system.

In 1984 Tanimura and co-workers (Takeuchi *et al* 1984) described the separation of enantiomers of isoleucine. They were able to devise a system in which the two enantiomers of isoleucine partitioned differentially in a solution of *n*-butanol-water containing copper(II) ions, *N*-*n*-dodecyl-L-proline and acetate buffer (pH5.5). The separation was dependent on the concentration of the chiral agent in the mobile phase. The work is significant in the development of droplet counter current chromatography in that it is the first to attempt the separation of the enantiomers of a particular compound. Previous separations simply utilised the differences in partitioning of solutes between the two phases. In this system additional solutes were added to the solvents in order to induce differences in partitioning. Oya and Snyder have reported the chiral separation of a carboxylic acid by DCCC (Oya and Snyder 1986) using an amino alcohol, (-)-(R)-2-aminobutanol as the stereospecific complexing agent.

Hermans-Lokkerbol and Verpoorte have reported the use of a pH gradient in separating a test mixture of alkaloids (Hermans-Lokkerbol and Verpoorte 1986). They used a system comprising chloroform-methanol-buffer (5:5:3) with the lower layer as the stationary phase. The pH of the mobile phase was decreased as the experiment progressed with the rationale that the alkaloids with higher pKa values would partition into the mobile phase before those with lower pKa's, and a separation would be achieved which utilised both the natural partitioning of the alkaloid in the solvent system as well as the partitioning induced by the change in pH as the experiment progressed. They also observed that ion-pair formation can influence the separation of alkaloids. Perchlorate was used as the counter-ion and a reduction in the concentration of perchlorate resulted

in greater partitioning into the aqueous phase and more rapid elution of the alkaloids. The indole alkaloids of some *Tabernaemontana* species in tissue culture were separated using the same solvent system and perchlorate as an ion-pair reagent (van der Heijden *et al* 1987)

Droplet counter current chromatography has been investigated for use in the determination of log *P* values (Gago *et al* 1986). The partition coefficient of a particular compound between water and *n*-octanol is used as a parameter in quantitative structure-activity relationships. The traditional shake-flask methods employed for determining these values are tedious and time consuming and the authors have proposed that droplet countercurrent chromatography be considered as an alternative.

4.2.3 Rotational locular counter current chromatography (RLCCC).

A description of rotational locular counter current chromatography was first proposed by Signer (1952). The apparatus typically consists of a glass tube of approximately 11mm internal diameter, which is divided into segments, or loculi, by discs (a commercially available apparatus uses Teflon discs), inserted into the tube and separated from one another by spacers. The discs have holes at the center to allow solvent to pass through. The column is loaded with stationary phase and inclined at an angle of 20° to 40°. The column is rotated and the mobile phase introduced at the appropriate end. The inclination of the tube allows the mobile phase to pass through the system without displacing the stationary phase. Partitioning of solutes occurs at each loculus and solutes are eluted according to their partition coefficient in the particular solvent system. With the commercially available apparatus, sixteen glass columns are mounted cylindrically about a rotational axis and joined in series with Teflon tubing (Snyder *et al* 1984). Snyder *et al* (1984) report that

equilibration at each locus is not achieved since an apparatus containing 592 loculi only generates between 250 and 300 theoretical plates.

RLCCC has not been used as extensively as droplet counter current chromatography or the rotating coil types of counter current chromatography, possibly because of the lower resolution obtained with this technique. Pieters and Vlietinck (1986) have used the method to fractionate the diterpene esters from croton oil. The diesters of phorbol were reasonably well separated from the triesters although baseline separation of individual compounds was not achieved. The solvent system used was petroleum ether-diethyl ether-methanol-water (18:2:15:0.75). Domon *et al* (1982 b) have separated the stereoisomers of norephedrine using RLCCC using 0.5M sodium hexafluorophosphate (pH4) as the stationary phase and 0.3M (*R,R*)-di-5-nonyl tartrate in 1,2-dichloroethane as the mobile phase.

Kolobow *et al* (1985) have described a development of RLCCC in which the loculi are moulded in sheets of polypropylene. They describe the apparatus as a "monolithic integrated flow circuit". The apparatus was operated in the conventional RLCCC mode, i.e. columns were rotated, and the performance was compared with two additional forms of agitation, gyration and oscillation. The efficiency of the apparatus in separating the N-2,4-dinitrophenyl derivatives of alanine and glutamic acid was better in the gyrating mode than in the oscillation mode. Simple rotational mixing was least efficient.

4.2.4 Coiled tube counter current chromatography.

Coiled tube counter current chromatography is used as an umbrella title for the different types of counter current chromatography developed by Dr Y. Ito and his colleagues at the laboratories of the National Institutes of Health, Bethesda, Maryland. The various types of chromatography in this class were developed from the mid 1960's until the present time and cover analytical separations on a microgram scale to preparative separations on a gram scale, using plastic or glass tubing of differing diameters wound onto a coil which may or may not be rotated in a centrifugal field. Apparatus using the technology of Dr Ito are now commercially available.

Ito and coworkers first observed that two immiscible solvents in a coiled tube could be segmented and made to undergo countercurrent flow (Ito *et al* 1966). The system they used worked on a micro scale and only minute quantities of their test mixture were separated. The technique generated an efficiency of between 2500 and 5000 theoretical plates which, at the time, was regarded as being comparable with gas chromatography (Ito and Bowman 1970). It was soon realised that placing the coiled tube in a centrifugal field improved the performance of the apparatus and that larger scale separations were possible (Ito and Bowman 1971). Ito called the apparatus a flow-through coil planet centrifuge. In earlier work, the coil did not rotate on its own axis but retained a constant orientation within the centrifugal field. The apparatus was later adapted so that the coil rotated about its own axis as well as in the centrifugal field. Sutherland and Sharpe (1976) discussed parameters relating to the solvents used in stationary coil planet centrifuge countercurrent chromatography. They recognised the need for solvents to have low viscosity and high interfacial tension for adequate performance.

The droplet size in the apparatus is important. If the droplets are too small emulsions form, resulting in carry over of the stationary phase. They estimated that the droplet size should be in the order of 0.1mm diameter. They also recognised that best conditions for separation occur when the phase volume ratio lies between 0.4 and 2 and that the operating temperature should be constant to within 0.5°C.

In the early 1980's Ito modified the apparatus to perform very much faster separations than had previously been achieved. He called the technique high speed countercurrent chromatography. Whereas the coil in the flow-through coil planet centrifuge was of one layer only, the high speed machine had a multi layer coil. Ito has reviewed the development of high speed countercurrent chromatography (Ito 1984).

Sutherland, Heywood-Waddington and Ito (1987) have differentiated three basic types of coiled tube countercurrent chromatography and have discussed the respective types of mixing of the two liquid phases. Synchronous coil planet centrifuge type of apparatus in which the coil does not rotate on its own axis and in which the acceleration field is consequently uniform, undergo a cascade type of mixing. One half of a turn of the coil is occupied by one phase and the other half by the other phase in much the same way as in the original fixed coiled tube experiment. (See Fig. 4.1)

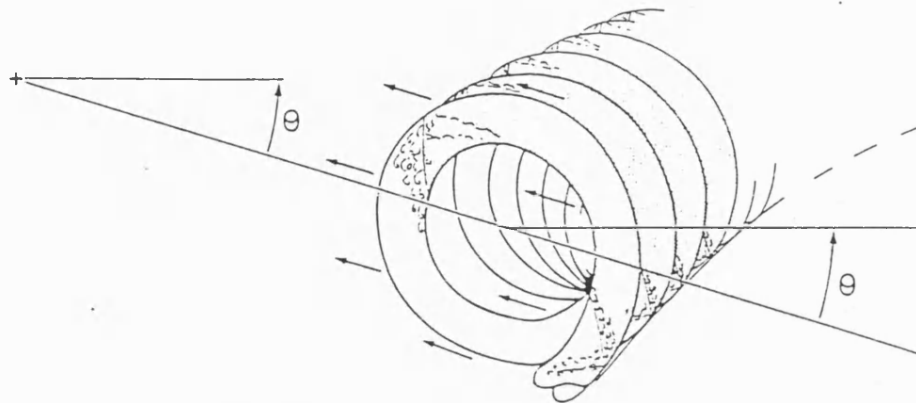


Fig. 4.1: Cascade mixing as occurs in a synchronous coil planet centrifuge. (from Sutherland et al 1987)

Epicyclic coil planet centrifuges, in which the coil is rotated about its own axis as well as in the centrifugal field, have a different phase distribution within the coil and a different means of mixing due to the fluctuating acceleration field (See Fig. 4.2).

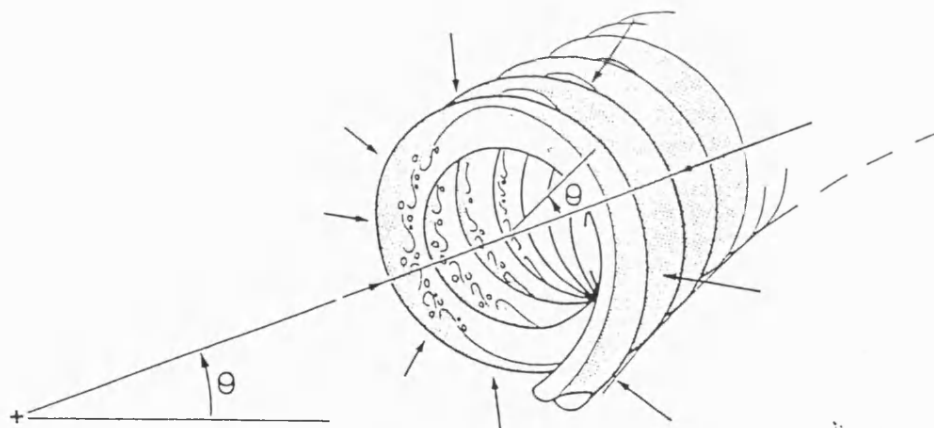


Fig. 4.2: Wave mixing as occurs in an epicyclic coil planet centrifuge (from Sutherland et al 1987)

The two phases occur in layers throughout the coil and a wave type of mixing occurs due to turbulence within the tube. The liquid within the tube is constantly forced to the tail of the coiled tube due to the Archimedes screw principle. With two immiscible liquids, the more dense liquid moves to the tail in preference to the less dense liquid, which is

displaced to the head.

The motion of a chosen point, z , on the coil is determined by the centrifugal radius and also on the radius of the coil. The ratio of the two radii is an important determinant of the contact of the two phases within the tube, and thus the retention of stationary phase with respect to mobile phase, i.e. the phase volume ratio, and the interfacial activity of the two liquids which determines the extent of mass transfer between the two phases.

Epicyclic coil planet centrifuges perform separations much faster than synchronous coil planet centrifuges, and are the basis of high speed countercurrent chromatography. Sutherland *et al* (1987) have compared the performance of the two in separating a mixture of macrolide antibiotics. The epicyclic coil plane centrifuge machine performed the separation in less than half the time taken on the synchronous coil planet centrifuge machine.

Epicyclic coil planet centrifuge systems, as with synchronous coil planet centrifuge systems require solvent systems with low viscosity, high density difference between the phases and high interfacial tensions (Sutherland *et al* 1987). Mixtures of organic solvents or organic solvents and water which form two immiscible phases are generally suitable for use in such apparatus. Double aqueous phase systems having increased viscosity, low density difference between the phases and low interfacial tension are not suitable. Such systems, however, are of interest in the separation of cells, organelles or cell components, and a machine which is capable of accommodating immiscible solutions of polymers would be of use for such a separation. Aqueous solutions of polyethylene glycol 6000 (5%)

and dextran T-500 (6%) are immiscible and can be buffered to a suitable pH and adjusted to any required tonicity (van Alstine *et al* 1985). A variation of such a system has been used in the separation of fragments of human deoxyribonucleic acid (Elles and Sutherland 1980). They used a toroidal flow coil centrifuge in which the plastic tubing was wound onto a flexible core which in turn was wound onto the coil and placed in the centrifuge. The coil was thus at right angles to the direction of rotation. Sutherland, Heywood-Waddington and Peters (1984) have described the apparatus, the mechanism of mixing and the application to the separation of the principal subcellular organelles of rat liver homogenates.

4.3 Computation of some countercurrent chromatography parameters.

4.3.1 Background and programming.

Keulemans (1957), in his discussion of the general theory of chromatographic separations, differentiates chromatography into four types on the basis of the distribution isotherm and whether the exchange process is thermodynamically reversible (See Fig 4.3). If the partition coefficient of a solute is independent of concentration - that is, if the plot of the concentration in the stationary phase against that in the mobile phase at constant temperature and pressure produces a straight line through the origin - then the distribution isotherm is linear. In a chromatographic or countercurrent flow situation, if the mass transfer coefficient is infinitely high, that is, if equilibrium between particle and fluid is immediate, then "ideal" chromatography conditions exist. Under such conditions, the width of a band of solute passing through a chromatography column would be constant throughout the column. Countercurrent distributions and countercurrent chromatography are "non-ideal" chromatographic processes as equilibrium of the solute between mobile and stationary phase is not immediate, the mass transfer coefficient is not infinitely high and the band width of a solute increases as it moves through the column.

linear ideal chromatography	linear non-ideal chromatography
non-linear ideal chromatography	non-linear non-ideal chromatography

Fig. 4.3 The four possible types of chromatography according to Keulemans (1957).

In many simple liquid-liquid systems the partition isotherm for solutes is linear. On this basis, it is possible to investigate some of the

parameters which affect the resolution generated during countercurrent chromatography, as this type of technique relies essentially on partitioning of solutes between two immiscible phases for separation. Factors which render the partition isotherm non-linear, such as very high concentrations of solutes, the use of varying concentrations of complexing agents or the use of solvent gradients during the development of the chromatogram would make theoretical prediction very much more complex, if not impossible.

Countercurrent chromatography can be considered as an extension of countercurrent distribution for purposes of the theoretical treatment. The factor of importance here is the length of column which is equivalent to one "stage" in the countercurrent distribution, that is, the length of column required for an effective exchange, n , to have occurred. The actual length of column for such an exchange in reality will be dependent on the solvent system in use, principally on the factors affecting mass transfer between the two phases. Such factors would include the molecular or atomic mass of a solute (the rate of movement of a solute in a liquid is inversely proportional to the mass of the solute), the viscosity of the liquids, the interfacial tension between the two phases and degree of turbulence at the phase interface.

The distribution of solutes within a countercurrent distribution is dependent on the partition coefficients of the individual solutes in the particular solvent system employed and the ratio of the volumes of the two phases. The fraction of a particular solute that is present in a specified tube can be calculated from Equation 12:

$$f_{n,r} = \left[\frac{n!}{r!(n-r)!} \right] (KR)^r \left[\frac{1}{1 + (KR)} \right]^n \quad \dots 12$$

With the use of a microcomputer, it is possible to perform a simulation of the binomial distribution with simple programming. However, even large mainframe computers are unable to handle the extremely large numbers generated by the factorials in equation 12, and for values of n greater than about 15, alternative methods of performing the simulation are needed. Cooke *et al* (1982) provide an algorithm for generating a binomial distribution but this method cannot operate above values of about 100.

In cases where the number of transfers is greater than 50, the binomial distribution approximates to a gaussian distribution, and a simpler equation for computational purposes can be used. Rothbart and Barford (1982) have compared the typical gaussian distribution to the binomial distribution and present the equation:

$$f_{n,r} = \left[\frac{1}{\sqrt{2\pi npq}} \right] \exp \left[\frac{-(r_{\max} - r)^2}{2npq} \right] \quad \dots 13$$

Using this equation, it is possible to program a microcomputer to calculate the fraction of solute present in each tube, r , after n transfers have occurred. Such a program in the computer language BASIC is presented in Appendix 1. The program requires the input of values for the partition coefficient of the solute, the phase volume ratio for the solvent system under examination, the number of stages in the apparatus and the number of transfers which the operator would like to simulate. The values for n , p and q are constant and the program calculates the probability of an event occurring at each stage in the distribution which is equivalent to the fraction of solute in each tube of the apparatus. The program was written to present the data in plot form as this is easily assimilated. The data could equally be presented as a printout of the values for f .

Additional programs based on the above can be written for two or more solutes so that the degree of separation of the components can be established. This is of use in situations where the number of transfers that can be generated on a particular apparatus is known and the effectiveness of that particular system in separating the components needs to be assessed.

If the partition coefficients of two solutes are known for a particular solvent system, then it is possible to compute the number of transfers that would be required to separate the solutes. The important factor in such a program is the definition of separation. In the normal distribution the mean is determined by the number of tries and the probability of the event occurring, i.e. $\text{mean} = np$. Two thirds of the distribution will lie within one standard deviation on either side of the mean, 95% will lie within two standard deviations and 99.74% will lie within three standard deviations. Baseline separation in chromatography is commonly defined as separation of two components by six times the standard deviation as derived from the normal or gaussian distribution. For components which are very similar, the standard deviation for each will be almost equal hence the use of the term "six times the standard deviation". For purposes of the theoretical argument, as a knowledge of the partition coefficient of each component is assumed, it is possible to calculate the standard deviation for each component, and separation can be defined as having occurred when the value for the mean plus three times the standard deviation for one component is less than the value for the mean minus three times the standard deviation for the other. Fig.4.4 gives a diagrammatic representation of a baseline separation of two components in terms of their respective means and standard deviations. The standard deviation from the mean for one component can be calculated from the

formula $s = \sqrt{npq}$. Appendix 2 lists a program for calculating the number of transfers required to separate two components of known partition coefficient from each other. Separation is defined as being three standard deviations from the mean. The program computes the relative positions of the leading and trailing edges of the distribution of a particular solute relative to a second solute, and prints out the value for the number of transfers required to achieve a 99.9% pure component.

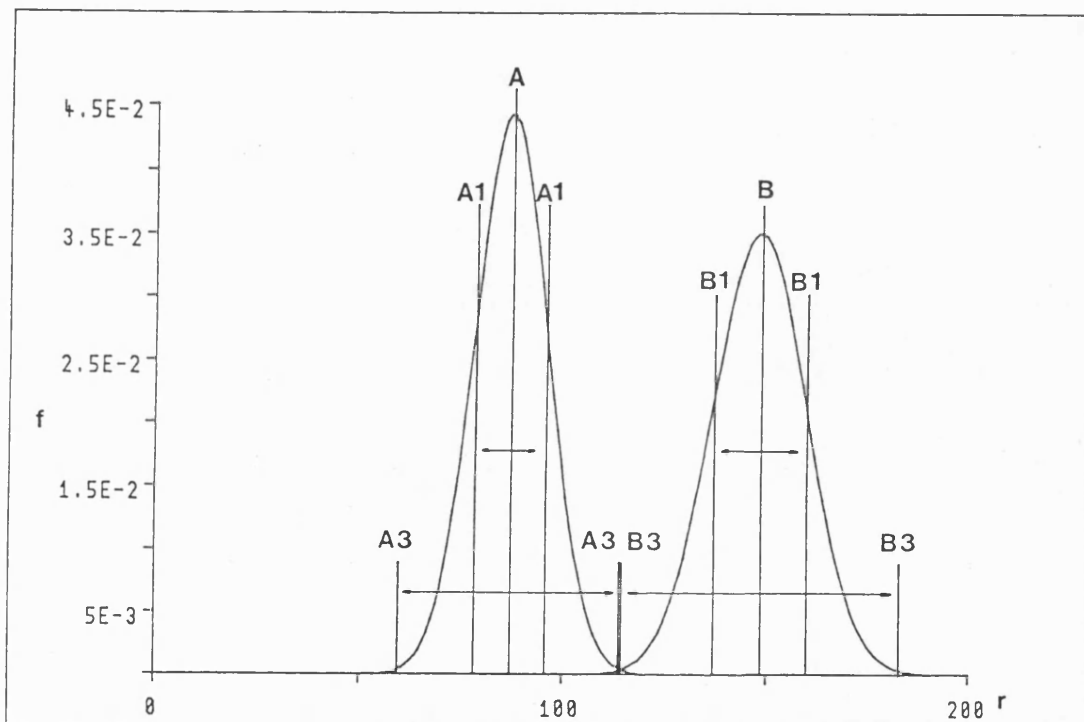


Fig 4.4: Separation of two peaks in terms of their respective means and standard deviations. A = mean for peak A, $A1$ = standard deviation for peak A, $A3 = 3 \times A1$, B = mean for peak B, $B1$ = standard deviation for peak B, $B3 = 3 \times B1$. $n = 1180$, $R = 0.08$, $K_A = 1$, $K_B = 1.8$.

Writing individual programs for each application can be tedious and "debugging" can be time consuming. A number of software packages for performing statistical calculations are commercially available which simplify the programming procedure considerably. One such package is "INSTAT" which was written in the computer department of Reading University for applications on BBC microcomputers. The package allows the operator to create a worksheet on the microcomputer. The worksheet is a series of columns each consisting of an equal number of rows. A facility

is provided for constants in the calculation, and strings, that is, alpha-numeric symbols which may be used in labelling but which are not involved in the calculations. The operator can define the values in a particular column. Data in a column may be entered piece by piece, or the column may represent a spread of numbers having an algebraic relationship to one another, for example, the values in a particular column may range from one to two thousand, in steps of fifty. The package allows the operator to perform a calculation on each value in the first column and to store the result in a second column. The entire calculation may be performed in one step, or, as in more complicated calculations, the equation may be divided up into smaller parts, and each part computed separately and the results combined at a later stage. The great advantage of the statistics package is that the various columns can be correlated with each other, or plotted against each other with minimal programming input by the operator. The package automatically selects a suitable scale for the plot, and allows the operator a choice of plotting formats.

4.3.2 Selection of a suitable solvent system.

The selection of solvent systems for use in countercurrent chromatography is commonly done on empirical grounds. Hostettmann (1980a) has proposed the use of a TLC system for choosing a solvent system, based on the R_f values of the compounds using the non-aqueous phase of the solvent system as the TLC solvent. This system would appear to be effective for the separation of glycosides and similar semi-polar compounds.

A more rational approach would be to use the partition coefficients of the compounds to be separated in a simulation of the process. Manipulating the partition coefficients by subtle changes in the solvent system should

allow the operator to select an appropriate system for the particular separation.

The computer program discussed in the above section could form the basis of a simulation, and the resolution of components under different conditions can be predicted. The optimum solvent system can therefore be selected for the separation.

4.3.3 Effect of various parameters on the separation of two solutes.

4.3.3.1 Partition coefficient.

A difference in partition coefficient is the most important parameter required for the separation of two solutes in countercurrent chromatography. The partition coefficient K of a solute is of primary importance in determining the value of p in the normal distribution analogy. Two solutes having the same partition coefficient can never be separated in that particular solvent system.

4.3.3.2 Phase-volume ratio.

The second component in the value of p is the phase-volume ratio, R . This has a profound effect on the speed and efficiency of separation of two solutes. Table 4.1 shows the effect of changing the phase volume ratio in the separation of two solutes, one having a partition coefficient of 1.0 and the second a partition coefficient of 1.3. The program listed in Appendix 2 was used to calculate the number of transfers, n , required to separate the two components.

Table 4.1: The number of transfers required to separate two components with partition coefficients of 1 and 1.3 respectively, at different phase-volume ratios.

Phase volume ratio R	Number of transfers, n .
1	2110
0.75	2108
0.5	2266
0.25	3032
0.1	5681

As the phase-volume ratio decreases, i.e. as the proportion of mobile phase to stationary phase decreases, the number of transfers required to separate the two components increases. This would suggest that optimum resolution is obtained at a phase-volume ratio of unity. However, as the phase-volume ratio decreases the retention of the solutes increases and for a given column length a greater number of transfers can be generated before the solute is eluted from the column. After 2110 equivalent transfers with a phase-volume ratio of 1, the mean for the solute with partition coefficient of 1 is at the 1055th "tube" and that of the solute with partition coefficient of 1.3 is at the 1193rd. If we assume that the apparatus consists of 1193 "tube" equivalents then after 2110 transfer equivalents the leading compound will have its maximum in the last tube. Table 2 shows the number of transfer equivalents required for the leading component to be found in the 1193rd "tube".

Table 4.2: The number of transfer equivalents required for the mean of the component with partition coefficient of 1.3 to be equal to 1193.

Phase-volume ratio	Number of transfers, n .
1	2110
0.75	2435
0.5	3059
0.25	4588
0.1	9942

A comparison with Table 4.1 shows that the number of transfers required to separate the two components at a phase-volume ratio of 0.1 is 5681, whereas the number of transfer equivalents required to bring the leading

component to the last "tube" is 9942. After 9942 transfer equivalents the two components will be separated to a much greater degree than after 5681. Metzger *et al* (1973) have recognised that reducing the phase-volume ratio to the smallest practicable value improves resolution. They also recognised the savings in solvent volume that this implies.

4.3.3.3 The spread of partition coefficient about unity.

It has been recognised that for best results in countercurrent chromatography, the partition coefficients of the solutes should lie between 0.2 and 5. Ideally, the partition coefficients of two solutes should be evenly spread about unity. If one plots the number of transfers required to separate two solutes, against the sum of the logarithms of the partition coefficients, or more correctly, the logarithms of the p values for the two solutes, a parabolic curve is the result, with the minimum value of n at zero, the log of 1. The data in **Fig 4.5** was obtained using the program in Appendix 2, to calculate the number of transfers, n , required to separate two solutes the ratio of whose p values is held constant. For simplification, the phase-volume ratio was set at one. Fig. 4.5 shows the relationship between the sum of the logarithms of p for two solutes and the number of transfers required to separate them.

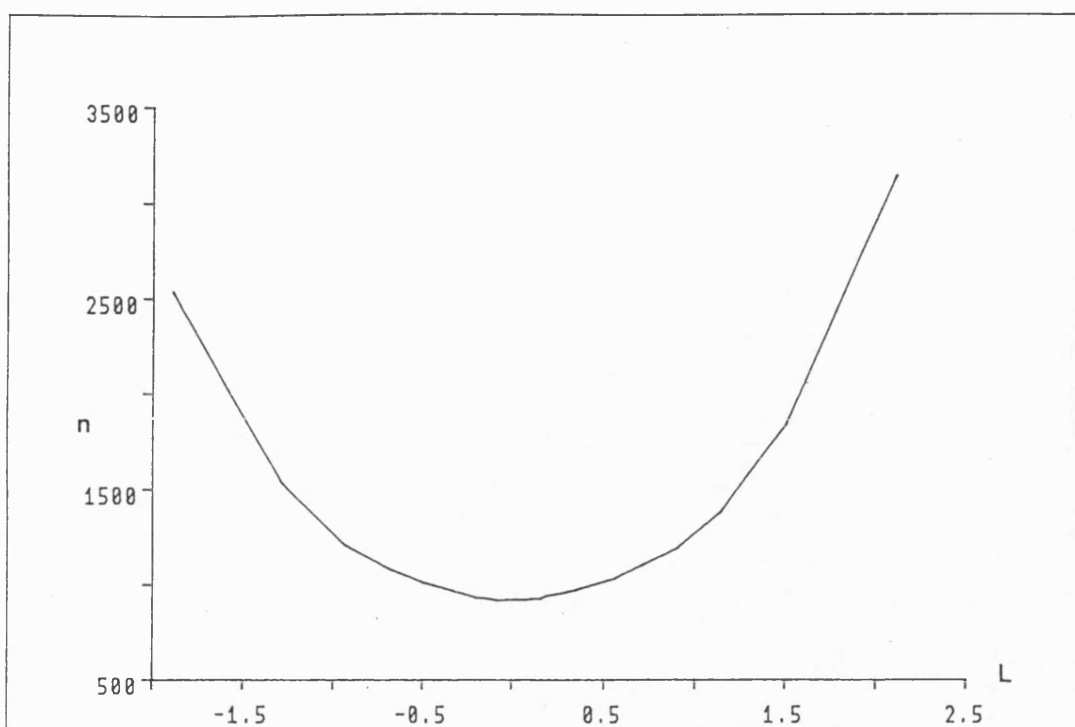


Fig 4.5: Plot of the number of transfers, n required to separate two components, against L , the sum of the logs of the two components. The ratio of the two components is 1.3.

4.3.4 Peak broadening as a function of n .

As a countercurrent distribution or chromatogram develops, the spread of a solute within the apparatus increases. Two factors influencing this broadening of the peak can be postulated. Lateral diffusion against the concentration gradient will have a peak broadening effect in countercurrent chromatography but not in countercurrent distribution, as each stage in the distribution is discrete from the next. In countercurrent chromatography lateral diffusion will vary with time so that in very slow processes, such as droplet countercurrent chromatography which may take several days for complete development of the chromatograph, the degree of lateral diffusion might be expected to be greater than in a faster process. Hostettmann *et al* (1984a) have proposed this factor as a reason for the increased efficiency of separation in some droplet countercurrent chromatography applications where the flow rate has been increased. The major reason for the broadening of peaks in countercurrent chromatography and distribution is, however, due to the broadening that

accompanies each successive transfer, n .

From the normal distribution model, 99.74% of a solute would lie between three standard deviations on either side of the mean, np . The standard deviation can be calculated from the equation $s = \sqrt{npq}$. p and q are constants, therefore the standard deviation and thus the spread of solute, is proportional to the square root of the number of transfers, n .

4.3.5 Peak shape in countercurrent chromatography.

Within a binomial, normal or gaussian distribution the peaks are symmetrical about the mean and it might be expected that countercurrent chromatography peaks would be symmetrical also. In practice it has been noted that the peaks are skewed (see chromatograms in experimental section). This skewing can be explained from the model for countercurrent chromatography.

In the binomial distribution, the peak shape and position is determined by the mean and standard deviation for the distribution. In countercurrent chromatography, the number of equivalent transfers, n , is continuously changing and thus the mean and standard deviations are continuously changing and therefore the peak shape will alter as the solute is eluted. The program in Appendix 1 plots the probability of an event occurring at any stage in a fixed series of stages, with each stage being one equivalent transfer in the countercurrent chromatography model. The plot gives the distribution of solute at a given moment. If the chromatographic process were to be halted at a particular moment and the distribution of solute calculated, the resulting plot would show symmetrical peaks. The elution chromatogram, however, does not show symmetrical peaks because the system is not stationary with respect to time. As the chromatogram

develops, n changes and with the change in n is a corresponding change in the mean and standard deviation. To obtain a simulation of the chromatogram, the program in Appendix 3 should be used. This program utilises the same formula as that in Appendix 1 except that the value for r , the tube number is held constant and n , the number of transfers, is varied. In a countercurrent chromatography situation, the number of tubes or tube equivalents is fixed, and it can be assumed that the value obtained on assaying the eluent is the same as for the last tube in the series. Fig. 4.6 represents a single solute simulation for a compound with known partition coefficient (1.8) and phase volume ratio (0.08), for a column of known length (115 "tubes"). The extent of the skewing can be measured by dropping a perpendicular from the top of the peak to the baseline. The skewness is defined in conventional chromatographic terms as the ratio of the distance from the perpendicular to the leading edge to that of the trailing edge at ten percent peak height.

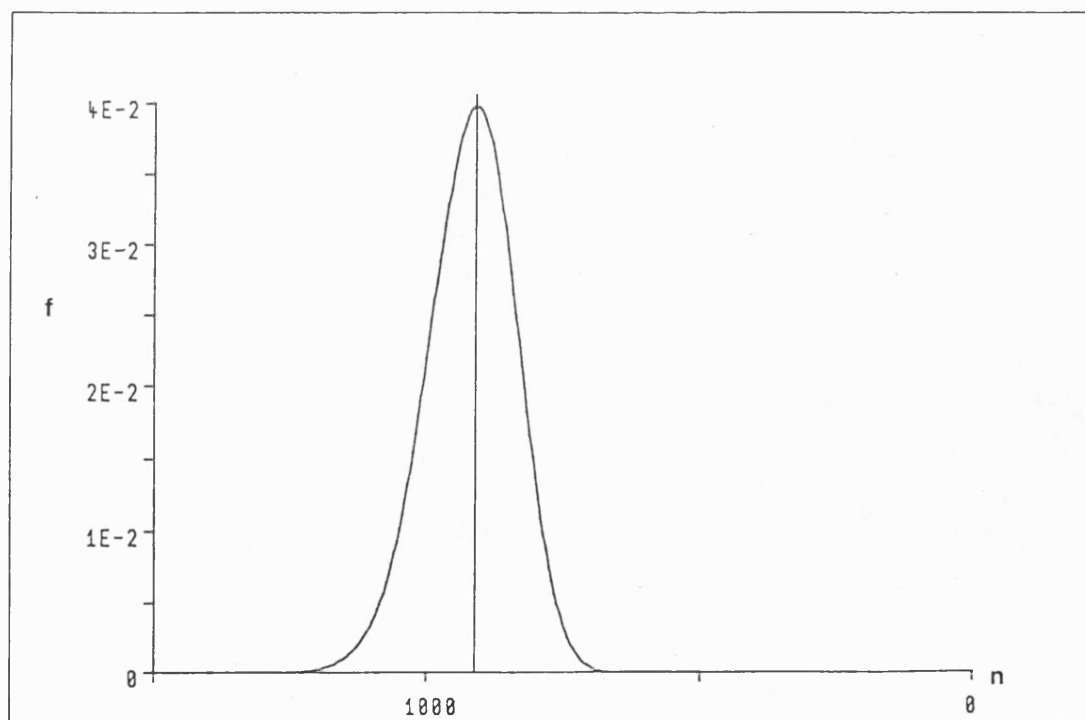


Fig 4.6: Single solute simulation showing peak skewing. $K= 1.8$, $R= 0.08$, $r= 115$.

Solute diffusion against the concentration gradient is also a factor in peak skewing. In very slow processes, such as droplet countercurrent chromatography, a peak may take a number of hours to be eluted and the degree of lateral diffusion at the tail end of the peak will be greater than at the leading end.

The linearity of the distribution isotherm has been assumed to be constant for purposes of this discussion. However, in practice, if the partition coefficient of the solute varied with concentration, then the peak will be skewed to the leading or trailing end depending on whether the partition coefficient changed in favour of the mobile or stationary phase.

4.3.6 Calculation of partition coefficient from retention.

The position of a compound within a countercurrent distribution or countercurrent chromatography apparatus is determined by the value of p for that compound and the number of transfers, n , which it has been subjected to. It should be possible to calculate the partition coefficient for a particular compound from the chromatographic trace provided the phase-volume ratio and the flow rate for the particular column is known.

The solvent front will be eluted after a time depending on the flow rate and the phase-volume ratio. The dead space, t_0 , which is the distance from the point of injection to the elution of the solvent front, can be calculated.

$$t_0 = \frac{\text{Volume of mobile phase (ml)}}{\text{Flow rate (ml/hr)}}$$

Any solute retained on the column will be retained to a degree which is proportional to its partition coefficient and the phase-volume ratio. The retention time for a compound, t_1 , can be calculated from t_0 .

$$t_1 = t_0/p$$

Conversely, the partition coefficient for a compound can be calculated from the retention time for the compound, provided the phase-volume ratio and the flow rate are known.

CHAPTER FIVE.

EXPERIMENTAL WORK.

5.1 Apparatus and materials.

Droplet countercurrent chromatography was carried out on a Buchi model B-670 DCCC machine, with glass tubes of 2.8mm internal diameter.

High speed counter current chromatography was carried out on an Ito planet coil centrifuge chromatography apparatus fitted with a 1.6mm internal diameter coil.

A Milton-Roy L.D.C. "Constametric" HPLC pump was used in conjunction with the Ito high speed countercurrent chromatography apparatus.

An L.K.B. 'Redirak' fraction collector with 100 15ml glass tubes was used.

Ultra-violet spectrophotometric post column detection was carried out on a Cecil Model 212 variable wavelength detector.

A Perkin-Elmer lambda-3 UV-visible spectrophotometer was used for absorbance measurements on selected solutions.

All solvents were of standard laboratory grade and were redistilled in glass before use. All solvents for droplet counter current chromatography were filtered through Whatman 0.45 μ m PTFE on polypropylene or 0.5 μ m cellulose acetate filters before use.

5.2 Droplet counter current chromatography - Methods.

5.2.1 Description of Buchi B-670 DCCC apparatus.

A schematic diagram of the apparatus is given in Fig. 5.1. The apparatus consists of a single piston reciprocating pump which pumps solvent from a reservoir to the columns via a pressure sensor, in-line filter and sample injection chamber. The pump is regulated by two controls, one which adjusts the stroke of the pump and the other the rate at which the plunger reciprocates. The pressure sensor is linked to the pump control and allows a variable maximum pressure to be set, above which the pump automatically cuts off. The maximum pressure at which the apparatus will operate is 40 bar. The in-line filter consists of a glass tube, 2.8mm internal diameter which is packed with glass wool. The sample chamber is of glass, and placed in an invertable mounting to allow selective injection and mixing prior to injection. Three sizes of sample chamber, 1ml, 5ml and 15ml, are provided for different applications. The various parts of the apparatus are linked with Teflon microbore tubing. The injection mechanism is linked to the columns via a coupling block which allows selection of the desired mode of operation, i.e. ascending or descending mode.

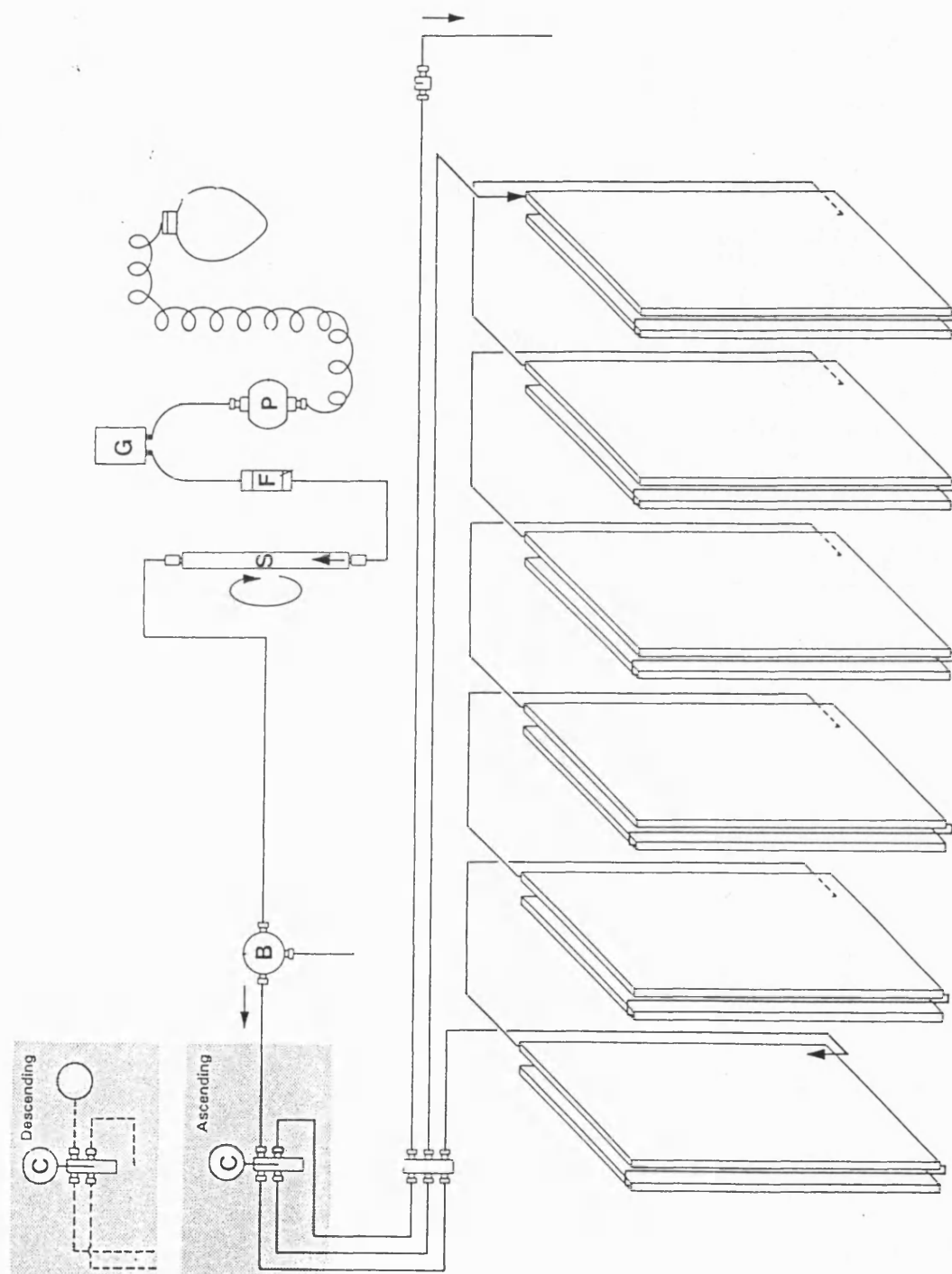


Fig 5.1: Schematic diagram of DCCC apparatus (from Buchi B-670 instruction manual). P= pump, G= pressure sensor, F= filter, S= sample tube, B= bleed point, C= column coupling block.

The columns are of glass, 2.8mm internal diameter and 40cm long. The columns are connected to one another with Teflon tubing. The Teflon tubing is connected to the glass columns via a coupling device which comprises a nut which fits on the outside of the column, a washer, spacer and bolt which are drilled to take the Teflon tubing which is flanged at the end. A capillary plate is located between the flanged Teflon tubing and the top of the glass column.

Columns are arranged in racks with 49 columns to each rack. The number of racks used in each experiment is variable. The racks are housed in the apparatus in an upright position along specially provided grooves and retaining lugs. A retaining bar is located at the front of the racks to prevent their accidental withdrawal.

5.2.2 Equipment modification.

5.2.2.1 Dampening mechanism.

A pressure dampening system was constructed from a 15ml glass tube attached at one end with teflon microbore tubing to a sealed glass tube (2.8mm i.d. x 5cm) and to a three way glass connector at the other. The dampener was fitted to the system via the glass connector between the pump and the pressure sensor.

5.2.2.1 Sample injection system.

The sample injection chamber was isolated from the system by a "Rheodyne" HPLC valve.

5.2.2.2 Detection system.

The outlet from the apparatus was fed into a glass two way splitting device to separate carried-over stationary phase from the mobile

phase. One outlet from the splitter was passed through a U.V. detector. The other bypassed the detector and was routed directly to the fraction collector or waste flask.

The modified apparatus is shown diagrammatically in Fig. 5.2.

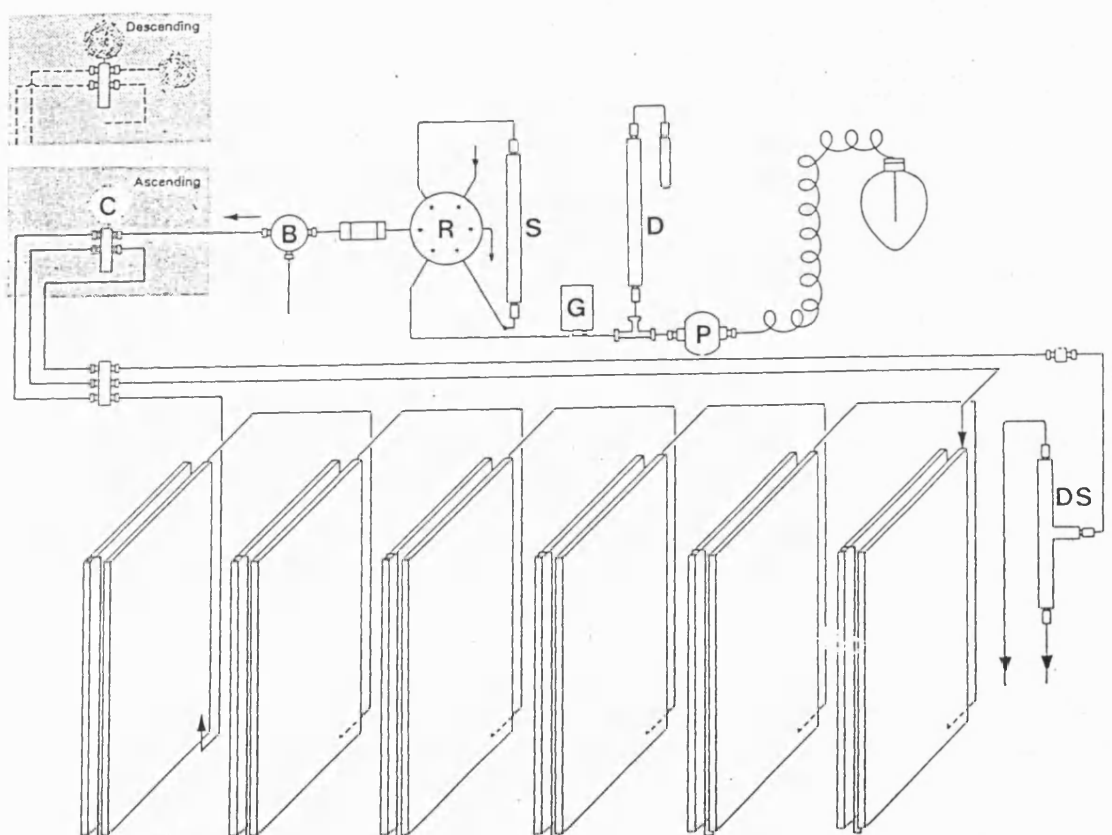


Fig 5.2: Modified DCCC apparatus. P= pump, D= dampening system, G= pressure gauge, R= "Rheodyne" valve, S= sample tube, B= bleed point, C= column coupling, DS= detector splitting device.

5.2.3 Development of suitable solvent systems.

A number of solvent systems for use in DCCC have been described in the literature and reviewed by Hostettmann and others (see chapter 4 for references). Prior to making up large volumes of solvents, a single tube apparatus was set up to test solvent systems for their ability to form suitable droplets. The apparatus consists of a 20ml glass syringe connected by teflon microbore tubing to a single DCCC tube, 2.7mm x 400mm in dimension held vertically in a retort stand. The tube was filled with the phase designated as stationary and the mobile phase drawn in at the appropriate end. Droplet formation was observed and possible problems with the phases noted.

The following solvent systems were made up and tested using the above apparatus.

System 1:	Hexane 100 Dichloromethane 30 Acetonitrile 70
System 2:	Hexane 36 Diethyl ether 4 Methanol 30 Water 1.5
System 3:	Hexane 80 Dichloromethane 20 Methanol 95 Water 5
System 4:	Hexane 100 Toluene 40 Ether 40 Methanol 100 Water 10
System 5:	Hexane 10 Ethyl acetate 5 Acetonitrile 10
System 6:	n-Hexane 100 Ethyl acetate 20 Diethylene glycol 20 Acetone 80 Water 1

System 7:	n-Heptane	50
	Acetone	10
	Methanol	40
System 8:	Chloroform	7
	Methanol	13
	Water	8
System 9:	n-Hexane	4
	Diethyl ether	8
	n-Propanol	3
	95% Ethanol	5
	Water	4

5.2.4 Use of model compounds to assess the performance of DCCC.

5.2.4.1 Partition coefficient determination.

Atropine sulphate and hyoscine hydrobromide were selected for use as model compounds. Both items were obtained from Sigma Chemical Company Ltd, Poole, Dorset.

A calibration curve for atropine sulphate was constructed to ascertain the linearity of response of the spectrophotometer. A series of dilutions in distilled water was set up and the absorbance at 254nm measured. A plot of concentration vs absorbance was drawn up.

100ml of solvent system 8 was prepared, using aqueous buffer (pH4.35 acetate buffer B.P.) and the two phases separated. 10ml of each phase was measured out and 15mg of atropine sulphate dissolved in the combined system. The two phases were allowed to settle completely and then separated. The absorbance of each layer was measured at 254nm against a blank reading for each phase after slight warming of the solution to ensure homogeneity. The partition coefficient was calculated as being the ratio of the absorbance of mobile phase (in this case the upper phase i.e. the aqueous-methanol phase) to the stationary phase.

5.2.4.2 Preparation of solvent systems.

Solvent systems for use in the DCCC were prepared in volumes of between 1L and 5L. The components were shaken together in a separatory funnel of suitable size and the system allowed to stand until the phases had separated. The two phases were stored separately in glass containers.

The buffer system was prepared by dissolving anhydrous sodium acetate (10g) in distilled water (300ml) and titrating with glacial acetic acid to pH4.35. The solution was diluted to 1000ml with distilled water and the pH checked again:

The solvent system chloroform:methanol:aqueous buffer pH4.35 was prepared and the lower phase selected as stationary phase.

5.2.4.3 Operation of DCCC apparatus.

The required number of racks of tubes was selected and the stationary i.e. lower phase pumped in at high pressure. The apparatus was set in the ascending mode to ensure that all air bubbles and residual solvent were flushed out from all parts of the apparatus. Once the stationary phase was in position, the mobile phase, i.e. the aqueous-methanol phase was introduced. The initial flow rate was kept to a minimum until the droplets were observed in the first tubes and then adjusted upwards until the maximum flow rate was achieved.

A stock solution of sample was prepared by dissolving the required amount of material in a portion of the mobile phase. Once the system was operating at the required flow rate the sample was loaded into the sample chamber via the "Rheodyne" loop valve and injected onto the column. The pressure cut off was set to a suitable level.

5.2.4.4 Monitoring of chromatograph eluent.

The eluent from the chromatograph was either collected in a fraction collector in 5ml aliquots or allowed to pass into a waste flask. A U.V. spectrophotometric detector was connected to the end of the column before the fraction collector with the flow splitting device in the appropriate position.

5.2.4.5 Comparison of the effect of column length on the retention of atropine.

A comparison was made using column lengths of 49, 98 and 294 tubes each with dimensions 2.7mm by 400mm. 1ml of a standard solution of atropine sulphate (20mg/ml) was injected onto each column in turn. The solvent system used was chloroform-methanol-pH4.35 acetate buffer 7:13:8 with the lower phase as stationary and the upper phase as mobile in the ascending mode. The flow rate was set at 16ml/hour. The eluent was passed through an ultra-violet spectrophotometric detector set at 254nm linked to a chart recorder operating at 1cm/hour. The apparatus was allowed to run until the injected compound had been completely eluted. The stationary phase was replenished between runs.

5.2.4.6 Use of beta-cyclodextrin as a chiral complexing agent in the attempted separation of the enantiomers of atropine.

Beta-cyclodextrin was dissolved in the mobile phase (2g/l) of the solvent system chloroform-methanol-pH4.35 acetate buffer and the experiment run using 294 tubes (2.7mm x 400mm) in the ascending mode at a flow rate of 16ml/hour. Atropine sulphate (20mg) dissolved in mobile phase (1ml) was injected onto the column after the flow rate was adjusted. The column eluent was passed through a U.V. spectrophotometric detector set at

254nm linked to a chart recorder operating at 1cm/hour.

5.2.4.7 Separation of atropine and hyoscine.

A stock solution of atropine sulphate and hyoscine hydrobromide (20mg/ml of each) was prepared and injected (1ml sample) onto a column of 294 tubes (2.7mm x 400mm) using the solvent system chloroform-methanol-pH4.35 acetate buffer in the ascending mode at a flow rate of 16ml/hour. The eluent was monitored on an ultra-violet spectrophotometer at 254nm connected to a chart recorder operating at 1cm/hour. The apparatus was allowed to run until no further peaks were eluted.

5.3 Ito high speed countercurrent chromatography.

5.3.1 Description and operation of apparatus.

The apparatus consists of a coil of teflon tubing (3.9m x 1.6mm) wound concentrically onto a metal reel. The coil is rotated about a central spindle and simultaneously about its own axis. The apparatus is driven by a variable speed electric motor at up to 800 revolutions per minute. The coil is counterbalanced by a metal weight with removeable brass and aluminium components as required for each particular situation. The inlet and outlet tubes on the apparatus are arranged in such a way as to avoid twisting of the tubes, thus removing the necessity for rotating seals. The coil and drive motor are contained in a metal housing. Fig.5.3 gives a diagrammatic representation of the apparatus.

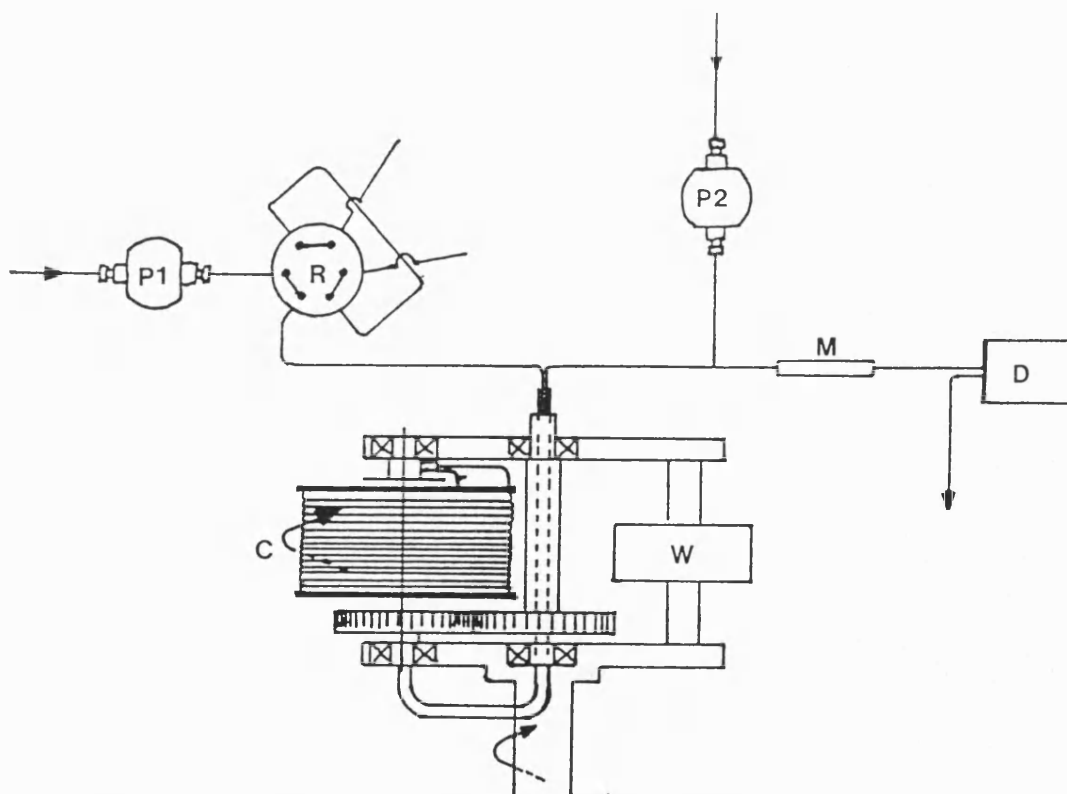


Fig 5.3: Schematic diagram of Ito high speed countercurrent chromatography apparatus. C= coil, W= counterweight, P1= main pump, R= "Rheodyne" valve and sample loop, P2= secondary pump, M= pre-detector mixing tube, D= detector.

An HPLC pump was used to fill the apparatus with stationary phase and to pump the mobile phase. The sample injection chamber was isolated from the pump-apparatus circuit by a "Rheodyne" loop valve.

The outlet of the coil is connected to a glass T-piece which allows the introduction of solvent from a second pump. The eluent from the T-piece leads to a mixing chamber consisting of a glass tube (2.7mm x 100mm) filled with glass beads (0.45-0.50mm diameter). The solvent is then fed to a variable wavelength U.V. spectrophotometric detector and then on to a fraction collector, if required.

The apparatus is prepared by filling the coil with stationary phase, taking care to exclude any air or residual solvent from the sample chamber and coil. The coil is then rotated to the required speed (up to 800 r.p.m.) and the mobile phase injected. Care must be taken to ensure that the orientation of the coil is correct for the selected solvent system i.e. if the more dense phase is used as the mobile phase, then the solvent must be pumped from head to tail and *vice versa*. When the solvent is being pumped, the second pump may be switched on and a solvent which is miscible with both phases pumped into the post column mixing chamber to facilitate UV detection.

5.3.2 Selection of solvent systems.

Solvent systems which are suitable for use in droplet countercurrent chromatography will generally be suitable for use in the Ito high speed countercurrent chromatograph. A simple test for solvent systems is to mix equal portions of the two phases in a stoppered tube, shake the tube vigorously for a few seconds and allow the contents to settle. If phase separation occurs within 30secs then the solvent system

is likely to be suitable for use in the Ito CCC.

Additional solvent systems prepared for the apparatus were:

System 1: *n*-Octanol 1
 Water 1

System 2: Chloroform 1
 Methanol 1
 Water 1

5.3.3 Use of model compounds to assess performance.

5.3.3.1 Retention of atropine sulphate.

The solvent system chloroform-methanol-pH4.35 acetate buffer (7:13:8) was prepared and the lower phase chosen as stationary phase. The counterbalance required was calculated by weighing 320ml of the stationary phase (387g) and adding the difference between this and the preset weight (320g) to the counterweight. The mobile phase was pumped at the rate of 5ml/min tail to head and the coil rotated at 800 r.p.m.. A sample of atropine sulphate (20mg) was dissolved in mobile phase (1ml) and injected onto the column. The second pump added methanol into the post column mixing chamber at a rate of 1ml/min. The eluent was monitored using a U.V. spectrophotometric detector at 254nm connected to a chart recorder operating at 12cm/hour. The apparatus was allowed to run until the sample peak had been completely eluted.

5.3.3.2 Separation of atropine from hyoscine.

The solvent system chloroform-methanol-pH4.35 acetate buffer (7:13:8) was prepared and the lower phase chosen as stationary phase. The apparatus was set up in the same manner as for the atropine retention experiment. A sample containing 20mg of each of atropine sulphate and hyoscine hydrobromide was prepared in 1ml of mobile phase and injected onto the column. The eluent was monitored on a UV detector at 254nm with a

chart recorder speed of 12cm/hour. The apparatus was allowed to run until both compounds had been eluted.

CHAPTER SIX

RESULTS AND DISCUSSION.

6.1 Droplet countercurrent chromatography.

6.1.1 Apparatus modification.

6.1.1.1 Dampening system.

Early attempts at using the Buchi DCCC apparatus were frustrated by the poor droplet formation in the tubes, especially in the first rack. A number of experiments failed because the flow rate of the machine had to be set to an extremely low level (below 3ml per hour) to allow reasonable droplet formation. It was thought that a possible reason for the poor performance was the pump on the instrument, which was a single piston reciprocating pump, which would deliver the solvent in pulses. At the peak of each pulse the droplet formation was impaired. A pulse-free pump driven from a nitrogen cylinder was attached to one rack of tubes and it was soon obvious that the problem was one of pulsing flow. The dampening system was designed using the end coupling pieces from broken tubes to ensure that good connections were obtained at the junctions. The pieces were made up in the University glass-blowing workshops. A 15ml sample injection chamber was used in-line in the dampening system and this proved adequate for the purpose.

Once the dampening system had been installed, droplet formation was much improved and higher flow rates were possible.

6.1.1.2 Sample injection system.

The original apparatus set-up loaded the sample through the pump. The solvent flask had an aspiration tip which could be removed, and this was placed in a test-tube containing the sample. When the sample volume had passed through the pump into the sample chamber, the aspiration tip was once again placed in the solvent flask.

One of the first separations attempted on the instrument was of a mixture of sterols. On passing the sample through the pump, it was noticed that the pump could not control the back-pressure from the tubes and on further investigation it was found that the non-return valves in the pump were stuck. It was noticed thereafter that the pump required a great deal of attention to keep the valves clean and the obvious reason for the problem was the injection of the sample through the pump. The isolation of the sample chamber using a "Rheodyne" injection valve meant that the sample could be injected directly onto the tubes without passing through the pump. Maintenance of the pump was much reduced once the alteration had been made. An additional advantage of the "Rheodyne" valve was that the flow of solvent was not interrupted in switching from sample injection onto the tubes to mobile phase.

6.1.1.3 Detection system.

Early attempts at monitoring the effluent from the machine using a UV detector were unsuccessful as it was not possible to achieve a stable baseline. On consideration, it was realised that the carry-over of stationary phase, although small, was probably the root of the problem. The splitting device was made up in the glass-blowing workshop using connection pieces from broken tubes, to ensure good connections. Once in place the mobile phase could pass through the detector as a clear

solution, with the carried-over stationary phase by-passing the detector and being fed directly to the fraction collector or waste flask. A ten second delay was used between the detector and the chart recorder as recommended by the manufacturer of the instrument, which further reduced any baseline perturbation.

6.1.2 Solvent systems.

The objective in testing various solvent systems was to find those which performed well in the apparatus. The required parameters were droplet formation and speed of flow. The single column apparatus for testing columns was found to be useful in that it allowed the assessment of a solvent system in a minimum of time using a small quantity of solvent (approximately 20ml). The use of a glass hypodermic syringe allowed the operator to test the solvent system in both the ascending and descending mode and could deliver a pulse free flow, which is essential for effective droplet formation and flow.

It was decided to test a variety of different solvent systems so that a range of suitable systems could be collated for use in different circumstances. A classification of solvent systems based on the polarity of the compounds which they would be suitable for would be ideal.

The following solvent systems were tested on the single column apparatus. In all instances the solvents which gave a positive test on the single tube apparatus worked well in the DCCC itself.

System 1: Hexane 100
 Dichloromethane 30
 Acetonitrile 70

The system had been reported in the literature and worked well in the DCCC. It is non-aqueous and might be suitable for the separation of fairly

non-polar compounds.

System 2: Hexane 36
 Diethyl ether 4
 Methanol 30
 Water 1.5

This system was an attempt to increase the polarity of the upper, mainly hexane layer, as the compounds being worked on (diterpene esters) partitioned preferentially into the methanol phase. Although two layers formed readily, and droplets were produced in the test they tended to aggregate in the column and formed slugs which then moved very slowly and displaced stationary phase from the system.

System 3: Hexane 80
 Dichloromethane 20
 Methanol 95
 Water 5

The system suffered from the same drawbacks as system 2. Slugs formed which did not flow through the stationary phase, but displaced it.

System 4: Hexane 100
 Toluene 40
 Diethyl ether 40
 Methanol 100
 Water 10

This system worked well in the DCCC. Good flow rates (up to 60ml/hour) were achieved. The drawback of the system was that toluene is not UV transparent and UV detection was therefore not possible. However, the system was considered to have potential in the separation of moderately polar compounds and that post column monitoring of the fractions collected could be done by thin layer chromatography. Toluene was included because it had been noted in column chromatography on silica that toluene sometimes gave a better separation of diterpene esters than hexane. The ether was included to increase the polarity of the hexane/toluene layer.

System 5: Hexane 10
 Ethyl acetate 5
 Acetonitrile 10

The system formed slugs and was not suitable.

System 6: *n*-Hexane 100
 Ethyl acetate 20
 Diethylene glycol 20
 Acetone 80
 Water 1

Good droplets were formed in the ascending mode only. The system was not considered suitable for further investigation because of the low flow rate due to diethylene glycol causing increased viscosity, and because of the potential problems in removing the diethylene glycol from the sample after use. Diethylene glycol was incorporated because it is sometimes used in partition preparative TLC for the purification of the more polar, often irritant diterpenes from Euphorbiaceae.

System 7: *n*-Heptane 50
 Acetone 10
 Methanol 40

Slugs formed in the column and consequently the system was not suitable.

System 8: Chloroform 7
 Methanol 13
 Water 8

This is probably the most widely used DCCC solvent system. Numerous applications in natural products have been reported (see for example Hostettmann's reviews on the subject) with this and variations on this system. Most of the experiments carried out in this work used a slight variation with the aqueous component buffered to pH4.35. The addition of the buffer salts did not appear to make any difference to the performance of the system in droplet formation or in flow rate.

System 9: Hexane 4
 Diethyl ether 8
 n-Propanol 3
 95% Ethanol 5
 Water 4

This system had been reported by Marshall and Kinghorn (1981) for the separation of phorbol and 4a-phorbol. On attempting to use the system it was found that it did not work due to the very low flow rate achieved and the slugs formed by the droplets.

6.1.3 Use of model compounds to assess performance.

6.1.3.1 Partition coefficient determination.

The solvent system chloroform-methanol-water 7:13:8 was chosen as it is a widely used one and it was felt that it should be suitable as a test system. None of the components were opaque to U.V. light at the wavelength suitable for the detection of the test compounds and the components were readily available. The aqueous component was modified to a pH4.35 acetate buffer as the pK_a of atropine is 4.35 and it was considered that this would give a partition coefficient for atropine close to 1. Hyoscine was the second test compound chosen. Atropine and hyoscine were selected as test compounds because the presence of an ionisable group in the molecules meant that the partition coefficients of the compounds could be manipulated by changing the pH of the system. The aromatic ring in both compounds meant that they would be readily detectable on a U.V. spectrophotometric detector. Atropine would also be a suitable test substance for the attempted separation of enantiomers.

Fig. 6.1 shows the calibration plot for the UV absorbance of atropine vs concentration in water at 254nm. The procedure was carried out to establish the range of concentrations between which the Beer-Lambert Law held true.

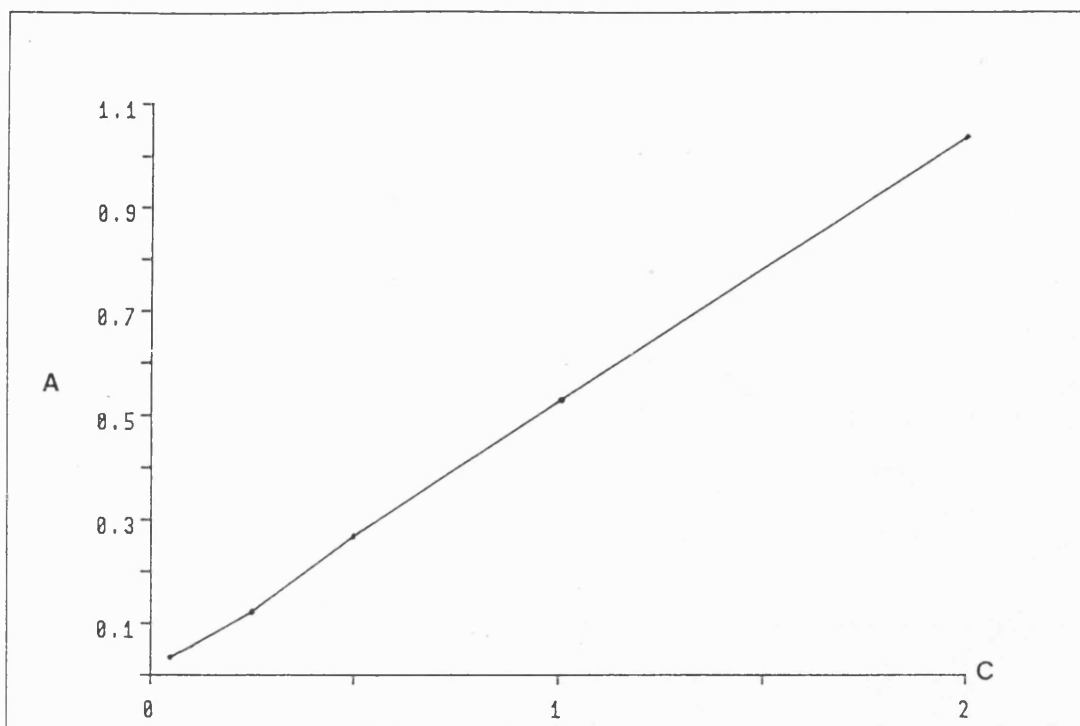


Fig 6.1: Calibration plot for the absorbance, A, at 254nm against concentration, C (mg/ml), for a range of concentrations of atropine sulphate.

Table 6.1.: Ultra-violet spectrophotometric absorbance readings and partition coefficients for atropine sulphate and hyoscine hydrobromide in the solvent system chloroform-methanol-pH4.35 buifer 7:13:8.

Absorbance upper layer	Absorbance lower layer	Partition coefficient (Aupper/Alower)	
Atropine 1.	0.783	0.462	1.7
Atropine 2.	0.470	0.251	1.87
Hyoscine 1.	0.605	0.633	0.96
Hyoscine 2.	0.336	0.322	1.04

The mean partition coefficient for atropine was taken as 1.3 and that for hyoscine as 1.0. The values for the partition coefficients were measured on separate occasions in different batches of solvent, and the differences between readings are probably due to small changes in the composition of the solvent system.

The partition coefficient for atropine was expected to be approximately 1 but was found to be closer to 2. That of hyoscine was very close to 1 and

it was decided that the solvent system would be suitable as the spread of partition coefficients was in the desired range with one of the components having a partition coefficient of 1.

6.1.3.2 Effect of column length on the retention of atropine sulphate.

The retention of atropine sulphate was observed for three different column lengths in the solvent system chloroform-methanol-pH4.35 acetate buffer 7:13:8. The phase-volume ratio was calculated at each column length and found to be 0.08.

Fig. 6.2 shows the chromatograms of the eluents of the three experiments. Fig. 6.2a is the trace for a column length of 49 tubes, Fig. 6.2b is for 98 column lengths and Fig. 6.2c for 294 tubes.

Table 6.2: Retention time and peak width for the retention of atropine in columns of differing length.

Column length (No. of tubes)	Retention time (hours)	Retention time (mm)	Peak width (mm)
49	3.4	34	29
98	6.7	67	48
29	21.2	212	71

There is a linear relationship between the retention of atropine and the column length. There is also a linear relationship between peak width and the square root of the column length. This is consistent with the theoretical statement that the peak width is proportional to the square root of the number of equivalent transfers, n , as an equivalent transfer is defined in terms of column length. If lateral diffusion is of primary importance in peak broadening, then it would be expected that the relationship between peak width and the square root of column length would not be closely adhered to. This does not appear to be the case, although more measurements would have to be made so that adequate statistical

testing could be done.

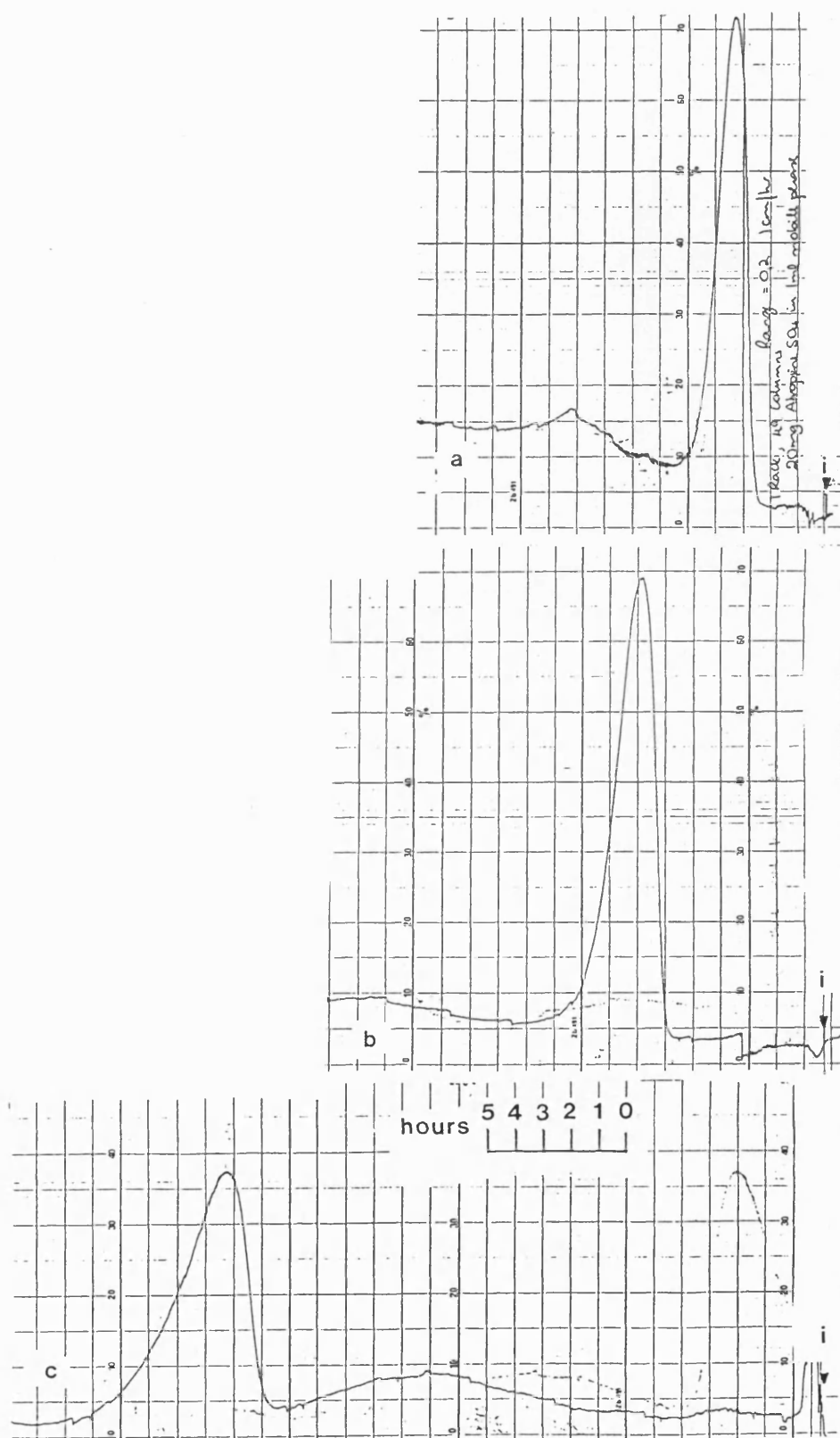


Fig 6.2: The effect of column length on the retention of atropine sulphate in DCCC. a = 49 tubes, b = 98 tubes, c = 294 tubes.

6.1.3.3 Use of beta-cyclodextrin as a chiral complexing agent in the separation of the enantiomers of atropine.

Cyclodextrins have been employed in High Performance Liquid Chromatography for the separation of enantiomers both as components of the mobile phase and when bonded to the solid support of the stationary phase (see for example Debowski *et al* (1983)). Davankov *et al* (1983) have reviewed the resolution of racemates by HPLC.

The major drawback of such systems is the cost. Cyclodextrins are expensive and cyclodextrin-bonded columns extremely so. It was decided to investigate whether cyclodextrins could be used in DCCC for chiral separations. If the experiment was successful then cyclodextrins could be incorporated into the stationary phase. This would require relatively little material which could be reused, thus avoiding the expense of a bonded column.

Atropine was again selected as a model compound because chiral specific complexation with cyclodextrin is frequently associated with compounds having a chiral centre close to an aromatic system. Propranolol and chlorpheniramine both have such chiral centers and the enantiomers of both compounds have successfully been resolved using cyclodextrin systems.

Beta-cyclodextrin was incorporated into the aqueous phase of the solvent system used in the previous experiment at the maximum possible concentration. The solvent was used as the mobile phase as this was the mode of operation of the experiment without cyclodextrin and it was necessary to compare the results of the two experiments. There is no reason why the aqueous phase should not be used as the stationary phase and the chloroform phase as mobile in a separate experiment. Fig. 6.3

shows the chromatogram of the cyclodextrin experiment (trace a) and compares it to the experiment without cyclodextrin (trace b). The retention of the peak from the cyclodextrin containing solvent was 21.7 hours compared with 21.2 hours for the cyclodextrin free solvent. The peak width for the cyclodextrin experiment was 70mm compared with 71mm for the previous experiment.

The incorporation of beta-cyclodextrin in the solvent system made no difference to either the retention or the peak width of the test compound. It may be that the concentration of cyclodextrin in the mobile phase was not high enough but it was not possible to dissolve more material in the solvent. Alternately, it may be that there is no enantiomer specific interaction between atropine and beta-cyclodextrin which may well be the case as one might expect peak broadening due to the interaction, but this is not observed.

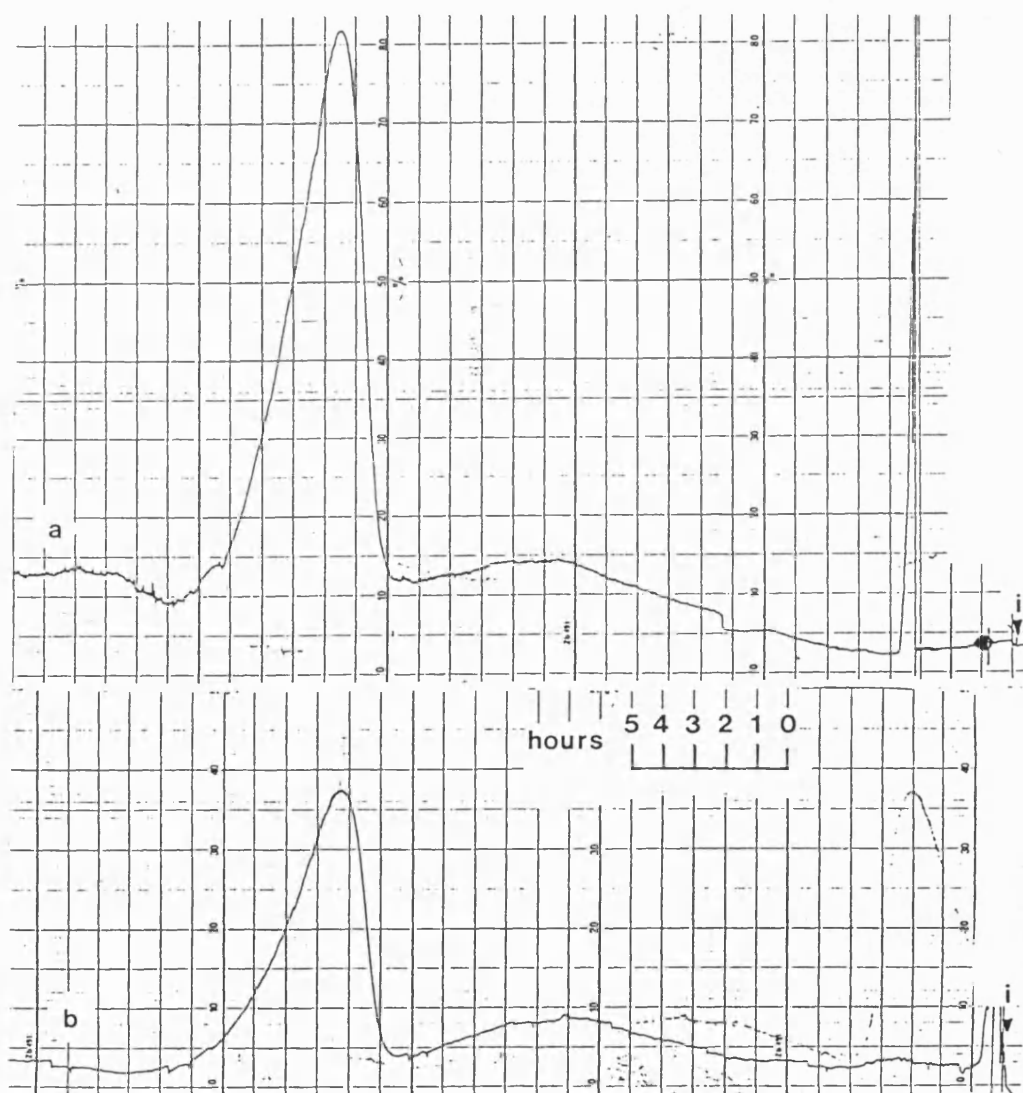


Fig 6.3: Comparison of the effect of the inclusion of beta-cyclodextrin in the mobile phase, on the separation of the enantiomers of atropine sulphate. a= mobile phase with cyclodextrin, b= mobile phase without cyclodextrin.

6.1.3.4 Separation of atropine from hyoscine.

A difference in partition coefficient is crucial if separation of two compounds is to be achieved by DCCC. As had been established the partition coefficients of atropine and hyoscine were 1.8 and 1 respectively. The two compounds should theoretically be reasonably well separated on a column length of 294 tubes.

Fig. 6.4 shows the chromatogram of the eluent for the experiment. As expected, atropine was eluted first after 22.5 hours with hyoscine coming off after 33 hours. There is a shoulder on the hyoscine peak due to an impurity in the hyoscine which has distorted the peak shape.

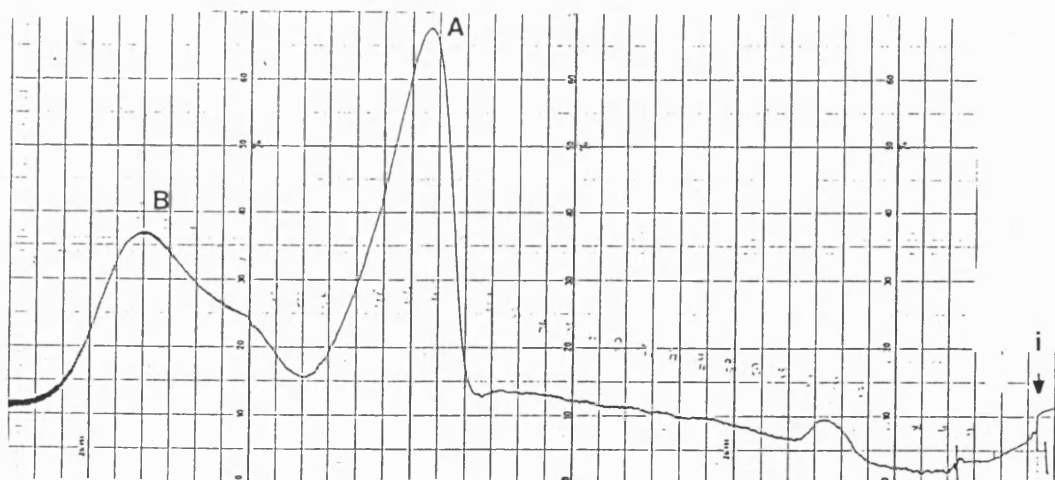


Fig 6.4: Separation of atropine sulphate and hyoscine hydrobromide by DCCC. A= atropine sulphate, B= hyoscine hydrobromide.

6.2 Ito high speed countercurrent chromatography.

6.2.1 Solvent systems.

Selection of suitable solvent systems for Ito high speed countercurrent chromatography presents far fewer problems than droplet countercurrent chromatography. Discreet droplet formation is not necessary and most solvent systems that form two phases will work. Shaking the two phases together in a stoppered tube is a useful, quick way of assessing the suitability of a particular system.

The *n*-octanol-water system was suggested in view of its use in log *p* determinations in Hansch analysis. Partition coefficients in this system have been published for a large number of compounds and if the system was found to be suitable for the Ito machine, then the published data could be used in the separation of mixtures of compounds. In the event the system was found to be unsuitable in that the mixture of water and octanol took longer than a minute for the phases to separate. A second drawback is that *n*-octanol has a high boiling point and removal of the solvent would be tedious in sample recovery.

Mixtures of chloroform, methanol and water were suitable for use in the apparatus. The same system as used in DCCC was applied to the Ito machine and the same model compounds were run on it.

6.2.2 Use of model compounds to assess performance.

6.2.2.1 Retention of atropine sulphate.

The same solvent system as for the DCCC was used with the same sample size. A stable baseline was achieved for the monitoring of the eluent by injecting additional methanol into the line immediately before the detector. An alternative methods for achieving the same result would be to pass the eluent through a heating block, which would increase the miscibility of the two phases. The total volume for the column was 320ml and the phase-volume ratio was calculated as 0.6. Fig. 6.5 shows the chromatogram of the eluent. The major peak for atropine was eluted after 46 minutes.

6.2.2.2 Separation of atropine and hyoscine.

A direct comparison was made between the Ito machine and the DCCC for speed of operation and resolution. The same solvent system was used as for the DCCC experiment and the same sample size injected onto the column. Fig. 6.6 shows the chromatogram of the eluent. Atropine was eluted after 48 minutes and hyoscine after 64 minutes. In DCCC the retention times were 22.5 and 33 hours respectively. The resolution obtained on the high speed machine was not appreciably better than on the DCCC but the time taken was considerably improved.

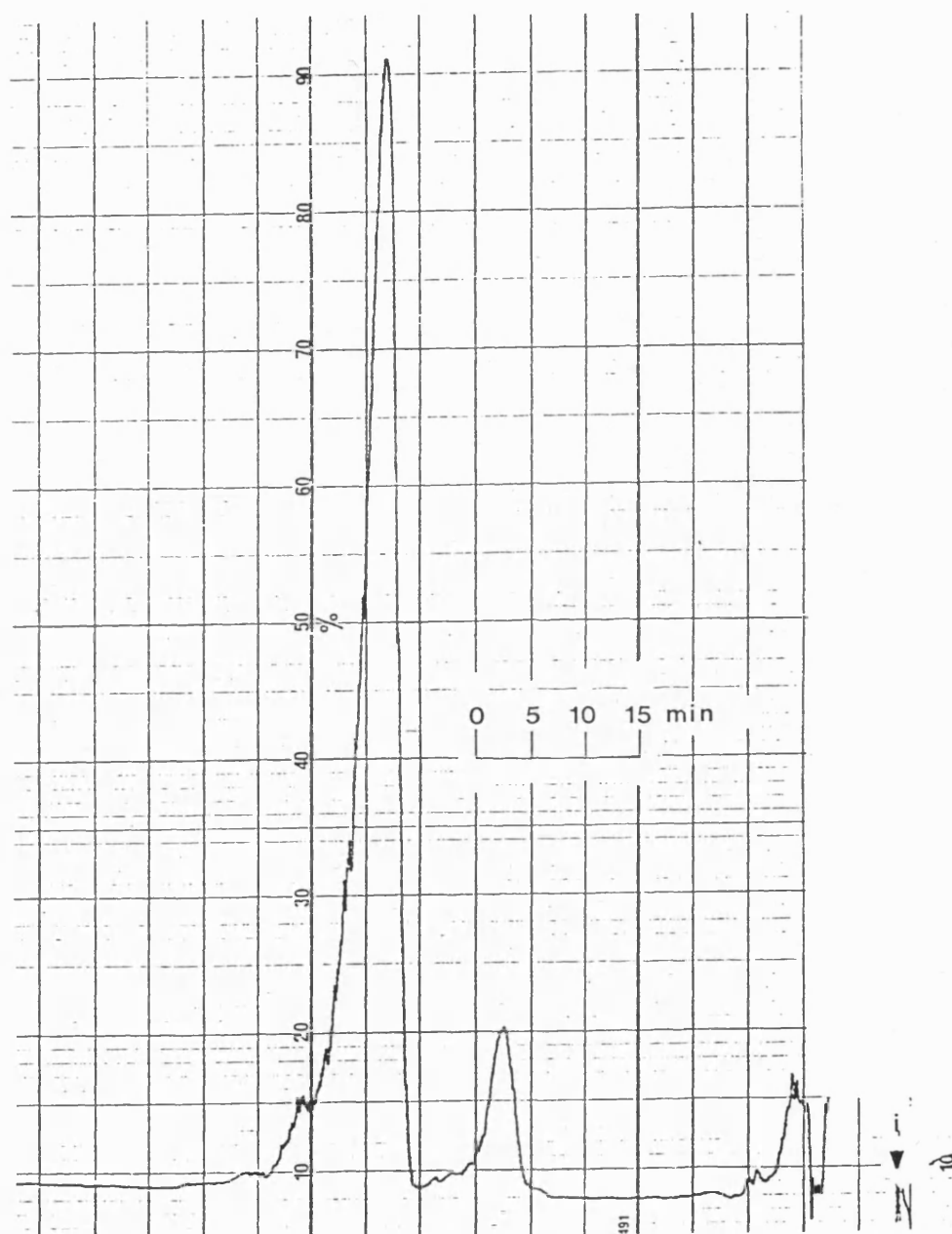


Fig 6.5: Retention of atropine sulphate in Ito high speed countercurrent chromatography.

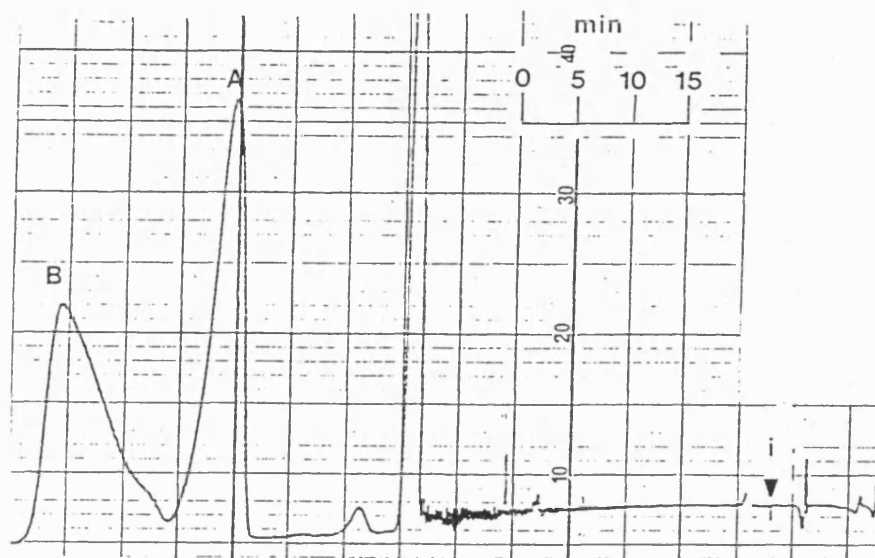


Fig 6.6: Separation of atropine sulphate, A, and hyoscine hydrobromide, B, by Ito high speed countercurrent chromatography.

6.3 Use of computer model to simulate solute resolution.

Using the partition coefficients and phase volume ratios for atropine and hyoscine for the two machines, the simulation program discussed in an earlier section is used to compare performance of the apparatus with the model.

It is difficult, if not impossible, to estimate the number of transfer stages, or "tubes", in either of the machines, as this would depend largely on the sample injection volume. It is assumed that this parameter would be fixed for a particular solvent system and sample injection volume, the determining factor being the rate of mass transfer between mobile and stationary phase.

The computer program in Appendix 2 was used to calculate the number of transfers required to separate the two components. The program also prints out the relative positions of the peaks and the value for the transfer number at which the two compounds are separated is taken for the length of the column. At this point, the leading compound would already have been eluted from the column, with the trailing compound still in the apparatus.

Using this program, the value obtained for the Ito high speed countercurrent chromatography apparatus was 190, and that for the DCCC was 115. This is in line with expectation, as it was shown earlier that the smaller the phase-volume ratio, the fewer the number of transfer stages required to obtain a separation.

When these values are used in the program (Appendix 4) to model the separation, the plots in Figs 6.7 and 6.8 are obtained. Fig 6.7 shows the simulation for DCCC. The actual chromatogram for the apparatus is shown

beneath it. Fig 6.8 shows the simulation for Ito high speed countercurrent chromatography with the chromatogram for the apparatus beneath it. The atropine peak is labelled A in each case and the hyoscine labelled B.

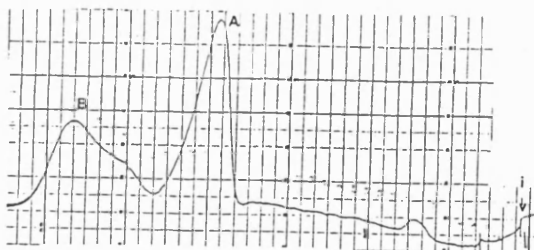
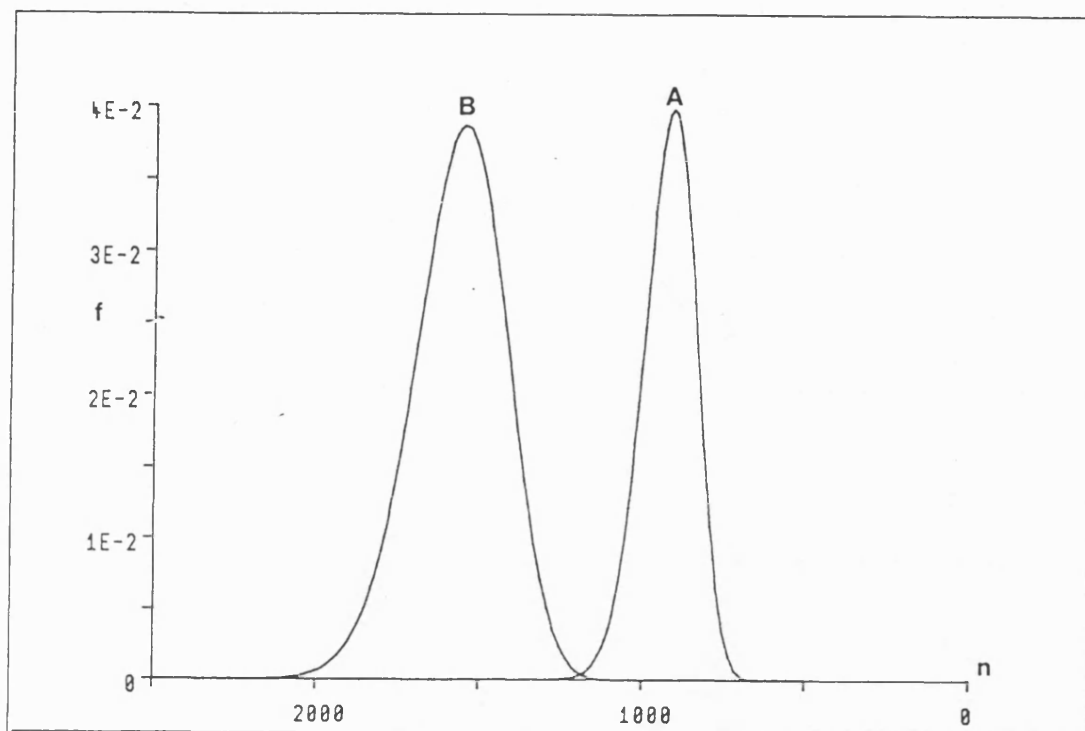


Fig 6.7: Comparison of the simulation of the separation of atropine sulphate and hyoscine hydrobromide with the actual separation by DCCC. The upper plot shows the simulation. The lower plot is the chromatogram.

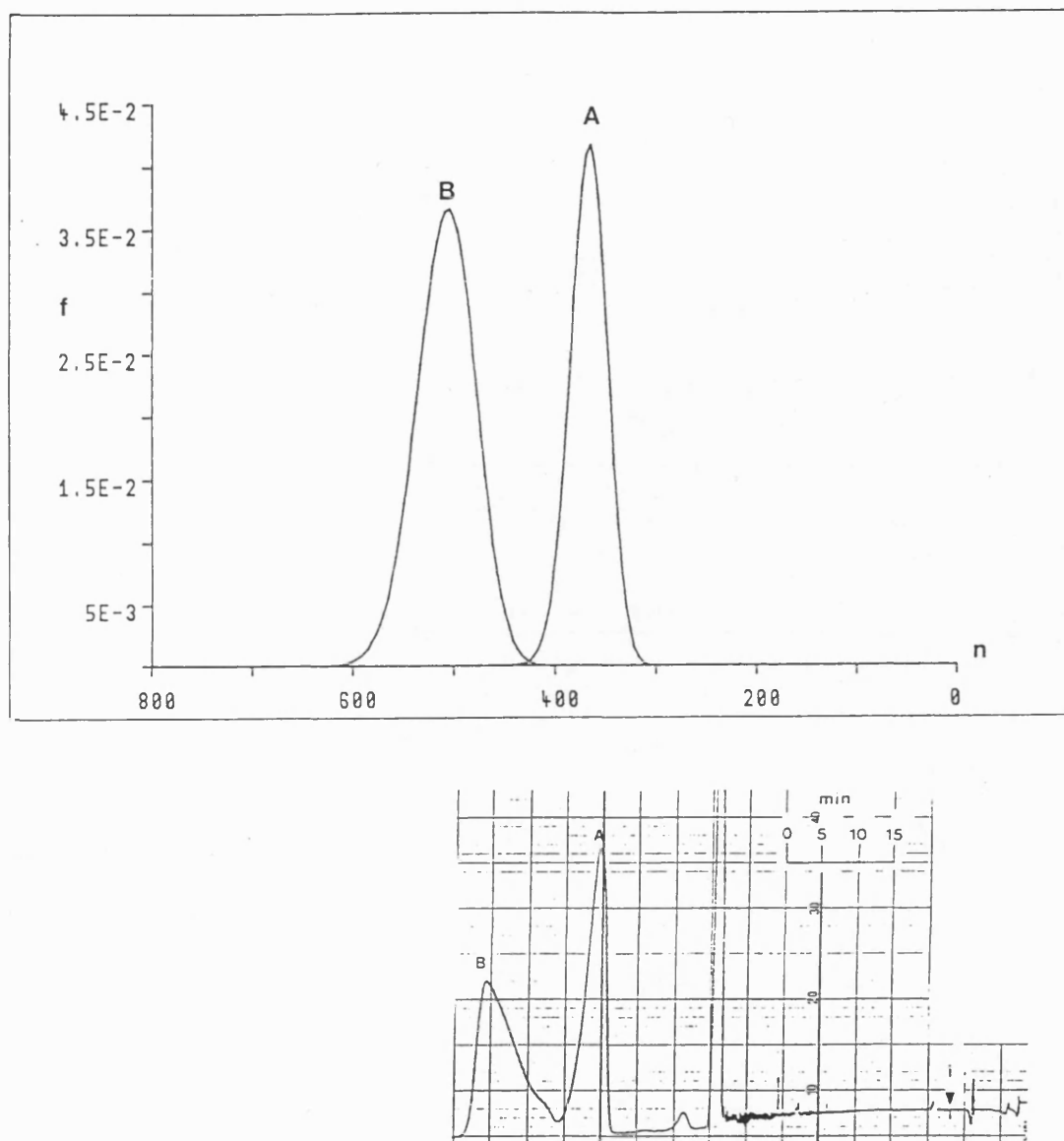


Fig 6.8: Comparison of the simulation of the separation of atropine sulphate and hyoscine hydrobromide with the actual separation by Ito HSCCC. The upper plot shows the simulation. The lower plot is the chromatogram.

From the plots it can be seen that the solutes are eluted in the predicted order. It is also apparent that the peak shape is essentially the same. The peaks are distorted and are not symmetrical as would be expected in a normal distribution.

Although the simulations do not exactly measure up to the chromatograms, the model is useful in that it allows the operator to predict whether resolution between two components will occur. Using the simulation it is possible to optimise the solvent system for a particular series of compounds on the basis of the partition coefficients for the compounds.

The simulation is also of use in giving an idea of the relative resolving power of DCCC and Ito high speed countercurrent chromatography. DCCC has the equivalent of about 115 transfers, whereas the high speed machine has 190. If the phase-volume ratio for the Ito machine was reduced to that of DCCC, higher resolution of solutes would be achieved.

6.4 Calculation of partition coefficient from retention time.

The retention time for any of the components could be predicted from the knowledge of partition coefficient, phase-volume ratio and flow rate, and, conversely, the partition coefficient for a particular compound could be predicted from the retention time. In the Ito high speed countercurrent chromatography apparatus, the flow rate was 5ml/min and the phase volume ratio 0.6. Using these data, it could be predicted that the solvent front would be eluted after 24min and that the atropine peak would be eluted after 46min. The atropine peak was in fact eluted after 48min. In the DCCC experiment, the solvent front would have been eluted after 3 hours, and the atropine peak after 23.8 hours. The actual elution time for the atropine was 22.5hours. In both cases there is good agreement between predicted and observed elution times.

The value of countercurrent chromatography for partition coefficient determinations is, however, uncertain. It has been established from a

theoretical point of view that ideal behaviour of the apparatus requires partition coefficients of between 0.2 and 5. Solutes with partition coefficients outside of this range will either be retained in the column for a long period of time, or will be eluted extremely quickly. One of the more important uses of partition coefficient determination is in quantitative structure activity relationships, and this is commonly quoted as a logarithm of the partition coefficient. The range of partition coefficients measureable by countercurrent chromatography lies within one unit only of the logarithmic scale and so the method would not be suitable for such determinations, as proposed by Gago (1986).

6.5 Separation of diterpenes using countercurrent chromatography.

Marshall and Kinghorn (1981) have reported the separation of phorbol and 4a-phorbol by DCCC. They used the solvent system hexane-diethyl ether-*n*-propanol-95% ethanol-water (4:8:3:5:4) with the lower phase as mobile phase on a Model-A DCC, Tokyo Rikakikai machine. Unfortunately, they do not report any further details of the apparatus such as the number of columns used or the internal diameter of the columns. The two compounds were not completely resolved and purification was completed by preparative liquid chromatography using an octadecylsilyl bonded phase column eluted with methanol-acetonitrile-water (1:1:6) which resolved the two compounds well.

Kinghorn has used DCCC in the fractionation of a number of extracts from other members of the Euphorbiaceae. Three 16-hydroxyingenol derivatives were isolated from *Euphorbia canariensis* (Lin et al 1983a). *Euphorbia hermentiana* latex yielded four 16-hydroxyingenol (Lin et al 1983b) and two 8-methoxyingol diterpenoids (Lin et al 1983c). In each fractionation, DCCC was used as a preliminary stage with the semi-pure fractions being further

purified using preparative TLC. Unfortunately the authors of these reports do not give adequate details of the DCCC experiments. Flow rate is not quoted, nor the number of tubes in the apparatus. However, it is apparent that the experiments were successful in enriching certain fractions with one of the components and this undoubtedly led to a more rapid isolation of the final product.

Pieters and Vlietinck (1986) used rotational locular countercurrent chromatography for the fractionation of croton oil. The components were not completely resolved, but phorbol diesters were separated from triesters, and short chained diesters were partially separated from long chained diesters. The method is of use in the early fractionation of the oil.

There are no reports of the the use of Ito high speed countercurrent chromatography in the purification of diterpenes from Euphorbiaceae or Thymelaeaceae. However, because of the dramatic increase in the speed of separation obtained on this instrument when compared with DCCC, it would be the preferred method for natural product purification. Separations could be accomplished within a working day, thus the technique could be used on the same time-scale as for column chromatography. The utilisation of a different mechanism of separation means that the resolution of closely related compounds would be facilitated.

6.6 Conclusions.

Countercurrent chromatography is a potentially useful tool for the fractionation of natural products. Its use should be evaluated in terms of the advantages and disadvantages associated with the two principal types of apparatus available, i.e. DCCC and Ito HSCCC.

Both instruments are expensive, currently in the order of thousands of pounds for the basic equipment. This does not include monitoring devices, nor, in the case of Ito high speed countercurrent chromatography, a pump for operating the apparatus. Consequently, a strong case must be made for the purchase of the equipment.

Droplet countercurrent chromatography is extremely slow in its operation. It is, however, cheaper than Ito high speed countercurrent chromatography to purchase. In the course of this work, a number of alterations had to be made to the instrument in order for it to operate effectively. Even after these alterations, high operator input and skill was required to obtain useful results.

Ito high speed countercurrent chromatography was easier to set up than DCCC. The selection of solvent systems that will work in the apparatus is less restrictive than with DCCC, and this gives the machine greater versatility. The increase in speed of operation of the machine over DCCC is the principal advantage. Separations can be achieved within a few hours.

The original advantages proposed by Hostettmann and others are valid. The lack of a solid adsorbant means fewer problems with regard to irreversible

adsorption. Compounds retained on the column can be recovered easily. Solvent consumption is greatly reduced in comparison with preparative HPLC.

The over-riding consideration has to be the resolving power of the technique, and at present this is not comparable with preparative HPLC. However, the development of the high speed machine has improved the performance of the apparatus considerably and further development work is in progress. An increase in the resolving power would make countercurrent chromatography indispensable in the natural products research laboratory.

APPENDIX 1

BASIC Program to calculate the fraction of solute present in each tube, r , after n transfers.

```

10 MODE0
20 INPUT"WHAT IS THE PARTITION COEFFICIENT?      "K
30 INPUT"WHAT IS THE PHASE VOLUME RATIO?        "Z
35 INPUT"HOW MANY TRANSFERS IN THE SIMULATION?    "n
40 R=n
50 p=K*Z/(K*Z+1)
60 Rm=n*p
70 r=Rm
80 q=1-p
90 s=SQR(n*p*q)
100 A=1/(s*SQR(2*PI))
110 B=-((Rm-r)^2)
120 C=2*n*p*q
130 FMAX=A*EXP((B/C))
132 FSC=INT(FMAX*(100+1))/100
140 PROCframe
150 FORr=1TOR
160 PROCcalc
170 PROCplot
180 NEXT
190 END
200 :
210 DEF PROCcalc
220 p=K*Z/(K*Z+1)
230 q=1-p
240 Rmax=n*p
250 s=SQR(n*p*q)
260 A=1/(s*SQR(2*PI))
270 B=-((Rmax-r)^2)
280 C=2*n*p*q
290 F=A*EXP((B/C))
300 ENDPROC
310 :
320 :
330 DEFPROCplot
340 xscale=1000/n
350 REMxo=xorig
360 PLOT5,xo+xscale*r,yo+yscale*F
370 ENDPROC
380 :
390 DEFPROCframe
400 yscale=600/FMAX
410 xo=200:yo=200
420 N=R
430 P=0.25*N
440 Q=0.5*N
450 T=0.75*N
460 S=N
470 VDU5
480 MOVExo,yo:DRAWxo,yo+600:MOVExo,yo:DRAWxo+1000,yo
490 MOVExo,yo:DRAWxo-20,yo:MOVExo,yo+150:DRAWxo-20,yo+150:MOVExo,yo+300:
    DRAWxo-20,yo+300:MOVExo,yo+450:DRAWxo-20,yo+450:MOVExo,yo+600:
    DRAWxo-20,yo+600: MOVExo,yo
500 MOVExo,yo:DRAWxo,yo-20:MOVExo+250,yo:DRAWxo+250,yo-20:MOVExo+500,yo:

```

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DRAWxo+500,yo-20:MOVExo+750,yo:DRAWxo+750,yo-20:MOVExo+1000,yo:
DRAWxo+1000,yo-20:MOVExo,yo
510 MOVExo-50,yo+10:PRINT"0":MOVExo-200,yo+160:PRINTFSC*0.25:
MOVExo-200,yo+310:PRINTFSC*0.5:MOVExo-200,yo+470:PRINTFSC*0.75:
MOVExo-200,yo+610:PRINTFSC
520 MOVExo-12,yo-50:PRINT"0":MOVExo,yo-50:PRINTP:MOVExo+250,yo-50:PRINTQ:
MOVExo+505,yo-50:PRINTT:MOVExo+750,yo-50:PRINTS
530 MOVExo-200,yo+300:PRINT"f":MOVExo+500,yo-100:PRINT"r"
540MOVExo,yo
550VDU4
560ENDPROC

```

APPENDIX 2.

BASIC program to calculate the number of transfers, n , required to separate two components of known partition coefficient.

```

10 MODE128
20 INPUT"WHAT IS THE PARTITION COEFFICIENT OF SOLUTE 1?" "K
30 INPUT"WHAT IS THE PARTITION COEFFICIENT OF SOLUTE 2?" "K2
40 INPUT"WHAT IS THE PHASE VOLUME RATIO?" "R
50 FORn=1TO100000
60 r=n
70 :
80 P1=K*R/(K*R+1)
90 RM1=INT(n*P1+0.5)
100 Q1=1-P1
110 S1=SQR(n*P1*Q1)
120 A=INT(RM1-(3*S1)+0.5)
130 B=INT(RM1+(3*S1)+0.5)
140 P2=K2*R/(K2*R+1)
150 Q2=1-P2
160 RM2=INT(n*P2+0.5)
170 S2=SQR(n*P2*Q2)
180 C=INT(RM2-(3*S2)+0.5)
190 D=INT(RM2+(3*S2)+0.5)
200 :
210 IF C>B GOTO240
220 IF A>D GOTO240
230 NEXT
240 PRINT"THE NUMBER OF TRANSFERS REQUIRED TO SEPARATE THE TWO COMPONENTS
IS "n
250 PRINT"95% OF THE SOLUTE WITH THE PARTITION COEFFICIENT"K" WILL BE
FOUND BETWEEN TUBES"A" AND"B
260 PRINT"THE MEAN POSITION FOR SOLUTE ONE IS AT TUBE NUMBER "RM1
270 PRINT"95% OF THE SOLUTE WITH THE PARTITION COEFFICIENT"K2" WILL BE
FOUND BETWEEN TUBES"C" AND"D
280 PRINT"THE MEAN POSITION FOR SOLUTE TWO IS AT TUBE NUMBER "RM2
290 END

```

APPENDIX 3.

INSTANT program to calculate the fraction of solute present at a particular point, r , for variable values of n . Data is presented as a plot of f vs n .

NOTE PROGRAM APPENDIX 3

NOTE K1=PARTITION COEFFICIENT

NOTE K2=PHASE VOLUME RATIO

NOTE K3=TUBE NUMBER (FIXED VALUE FOR r)

NOTE X1=VALUES FOR N

$K4 = K1 * K2 / (1 + K1 * K2)$

$K5 = 1 - K4$

$X2 = 2 * K4 * K5 * X1$

$X3 = -((X1 * K4 - K3)^2)$

$X4 = (1 / \text{SQR}(\text{PI} * X2)) * \text{EXP}(X3 / X2)$

LINX4 1 1 1

PLOTX4 X1

APPENDIX 4.

INSTANT program to simulate the separation of two components of known partition coefficient.

NOTE PROGRAM APPENDIX 4

NOTE K1=PARTITION COEFFICIENT 1

NOTE K2=PARTITION COEFFICIENT 2

NOTE K3=PHASE VOLUME RATIO

NOTE K4=TUBE NUMBER (FIXED VALUE FOR r)

NOTE X1=VALUES FOR N

NOTE SI=TITLE FOR PLOT

$K5 = K1 * K3 / (1 + K1 * K3)$

$K6 = 1 - K5$

$K7 = K2 * K3 / (1 + K2 * K3)$

$K8 = 1 - K7$

$X2 = 2 * K5 * K6 * X1$

$X3 = -((X1 * K5 - K4)^2)$

$X4 = (1 / \text{SQR}(\text{PI} * X2)) * \text{EXP}(X3 / X2)$

$X5 = 2 * K7 * K8 * X1$

$X6 = -((X1 * K7 - K4)^2)$

$X7 = (1 / \text{SQR}(\text{PI} * X5)) * \text{EXP}(X6 / X5)$

$X8 = X1 * (-1)$

LINX4 1 1 1

LINX7 1 2 1

PLOTX4 X7 X8;TITS1

APPENDIX 5.

Details of crystal structure determinations.

The crystal structure determinations were undertaken by Dr K. Molloy and Dr M. Mahon in the School of Chemistry, University of Bath. Data was collected on a Hilger and Watts Y290 Automatic 4-circle diffractometer using Mo- $k\alpha$ radiation ($\lambda=0.71069\text{\AA}$). Both structure were solved by direct methods using SHELX86, (G.M. Sheldrick, SHELX86 a computer program for crystal structure determination, University of Goettingen 1986).

Hydrogen atoms were included in positions of fixed geometry and all atoms were refined isotropically.

Sy 215/1.

A crystal with approximate dimensions $0.5\times0.4\times0.45\text{mm}$ was used for data collection. It crystallised in the orthorhombic space group $P2_1 2_1 2_1$ with cell dimensions $a=11.514(4)\text{\AA}$, $b=16.521(7)\text{\AA}$, $c=17.695(5)\text{\AA}$. After least squares refinement, the final R-value was 8.66.

An10.

A crystal with approximate dimensions $0.3\times0.3\times0.3\text{mm}$ was used for data collection. It crystallised in the orthorhombic space group $P2_1 2_1 2_1$ with cell dimensions $a=6.442(4)\text{\AA}$, $b=14.015(4)\text{\AA}$, $c=28.691(9)\text{\AA}$. After least squares refinement, the final R-value was 9.18.

REFERENCES

- Abo, K.A. 1982
Ph.D. Thesis, University of London.
- Adolf, W.; Hecker, E. 1980
Tetrahedron Lett. 21 2887-2890
New irritant diterpene-esters from roots of *Stillingia sylvatica* L.
(Euphorbiaceae).
- Adolf, W.; Hecker, E. 1984
J. Nat. Prod. 47 (3) 482-496
On the active principles of the spurge family, X. Skin irritants,
cocarcinogens, and cryptic cocarcinogens from the latex of the manchineel
tree.
- Aitken, A. 1986
"The biochemical mechanism of action of phorbol esters."
Naturally Occurring Phorbol Esters. Ed. Evans, F.J. pg 271-288
C.R.C. Press, Boca Raton, Florida.
- Aitken, A. 1987
Bot. J. Linn. Soc. 94 (1/2) 247-263
The activation of protein kinase C by daphnane, ingenane and tiglane
diterpenoid esters.
- Alvin, K.L. 1987
Annals of Botany 59 579-591
Leaf anatomy of *Androstachys johnsonii* Prain and its functional
significance.
- Anjaneyulu, A.S.R.; Rao, M.N. 1980
Phytochemistry 19 (6) 1163-1169
Elaeodendrol and Elaeodendradiol, new nor-triterpenes from *Elaeodendron
glucum*.
- Baarschers, W.H.; Horn, D.H.; Johnson, L.-R.F. 1962
J. Chem. Soc. 4046-4055
The structure of some diterpenes from Tambootie wood, *Spirostachys
africana*.
- Bauer, R.; Tittel, G.; Wagner, H. 1983
Planta Medica 48 10-16
Isolation and detection of phorbol esters in Crotonoil with HPLC. A new
method for diterpene ester-screening in Euphorbiaceae.
- Becker, H. 1984
Fresenius Z. Anal. Chem. 318 225-227
Non-aqueous systems for droplet-counter-current chromatography (DCCC).
- Becker, H.; Reichling, J.; Hsieh, W-C. 1982
J. Chromatography 237 307-340
Water-free solvent system for droplet counter-current chromatography and
its suitability for the separation of non-polar substances.
- Berenblum, I. 1941
Cancer Research 1 44-48
The cocarcinogenic action of Croton resin.

- Betancor, C.; Friere, R.; Gonzalez, A.G.; Salazar, J.A.; Pascard, C.; Prange, T. 1980
Phytochemistry 19 1989-1993
Three triterpenes and other terpenoids from *Catha cassinoides*.
- Blumberg, P.M.; Nakadate, T.; Warren, B.S.; Dell'Aquila, M.; Sako, T.; Pasti, G.; Sharkey, N.A. 1987
Bot. J. Linn. Soc. 94 (1/2) 283-292
Phorbol esters as probes of the modulatory site on protein kinase C - an overview.
- Branch, S.K.; Casy, A.F. 1988
Progress in Medicinal Chemistry In press.
Applications of modern high-field NMR spectroscopic techniques to medicinal chemistry.
- Borris, R.P.; Cordell, G.A. 1984
J. Natural Products 47 (2) 270-278
Studies of the Thymelaeaceae II. Antineoplastic principles of *Gnidia kraussiana*.
- Compton, R.H. 1966
J. South African Botany Supp. Vol. VI
An annotated check list of the flora of Swaziland.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984a
Tetrahedron Letters 25 (34) 3773-3776
Five ingol esters and a 17-hydroxyingenol ester from the latex of *Euphorbia kamerunica*. Assignment of esters using ^{13}C N.M.R. methods.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984b
J. Chem. Research (S) 366-367
A reassessment of the relative stereochemistry of ingol tetra-acetate.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984c
J. Chem. Res. (S) 368-369
A novel 19-hydroxyingenol ester from the latex of *Euphorbia poisonii*; Specification of ester linkages using NMR methods, including two-dimensional long-range $^{13}\text{C}/^1\text{H}$ chemical shift correlation.
- Conway, W. 1984
Liquid Chromatogr. 2 (5) 368-376
Recent applications of countercurrent chromatography.
- Conway, W.D.; Ito, Y. 1985
J. Liquid Chromatography 8 (12) 2195-2207
Resolution in countercurrent chromatography.
- Cooke, D.; Craven, A.H.; Clarke, G.M. 1982
Basic Statistical Computing
Edward Arnold, London.
- Davankov, V.A.; Kurganov, A.A.; Bochkov, A.S.
Advances in Chromatography 22 71-116
Resolution of racemates by high-performance liquid chromatography.

- Betancor, C.; Friere, R.; Gonzalez, A.G.; Salazar, J.A.; Pascard, C.; Prange, T. 1980
Phytochemistry 19 1989-1993
Three triterpenes and other terpenoids from *Catha cassinoides*.
- Blumberg, P.M.; Nakadate, T.; Warren, B.S.; Dell'Aquila, M.; Sako, T.; Pasti, G.; Sharkey, N.A. 1987
Bot. J. Linn. Soc. 94 (1/2) 283-292
Phorbol esters as probes of the modulatory site on protein kinase C - an overview.
- Branch, S.K.; Casy, A.F. 1988
Progress in Medicinal Chemistry In press.
Applications of modern high-field NMR spectroscopic techniques to medicinal chemistry.
- Borris, R.P.; Cordell, G.A. 1984
J. Natural Products 47 (2) 270-278
Studies of the Thymelaeaceae II. Antineoplastic principles of *Gnidia kraussiana*.
- Compton, R.H. 1966
J. South African Botany Supp. Vol. VI
An annotated check list of the flora of Swaziland.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984a
Tetrahedron Letters 25 (34) 3773-3776
Five ingol esters and a 17-hydroxyingenol ester from the latex of *Euphorbia kamerunica*. Assignment of esters using ^{13}C N.M.R. methods.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984b
J. Chem. Research (S) 366-367
A reassessment of the relative stereochemistry of ingol tetra-acetate.
- Connolly, J.D.; Fakunle, C.O.; Rycroft, D.S. 1984c
J. Chem. Res. (S) 368-369
A novel 19-hydroxyingenol ester from the latex of *Euphorbia poissonii*; Specification of ester linkages using NMR methods, including two-dimensional long-range $^{13}\text{C}/^1\text{H}$ chemical shift correlation.
- Conway, W. 1984
Liquid Chromatogr. 2 (5) 368-376
Recent applications of countercurrent chromatography.
- Conway, W.D.; Ito, Y. 1985
J. Liquid Chromatography 8 (12) 2195-2207
Resolution in countercurrent chromatography.
- Cooke, D.; Craven, A.H.; Clarke, G.M. 1982
Basic Statistical Computing
Edward Arnold, London.
- Davankov, V.A.; Kurganov, A.A.; Bochkov, A.S. 1983
Advances in Chromatography 22 71-116
Resolution of racemates by high-performance liquid chromatography.

- Debowski, J.; Jurczak, J.; Sybilska, D. 1983
Journal of Chromatography 282 83-88
Resolution of some chiral mandelic acid derivatives into enantiomers by reversed-phase high-performance liquid chromatography via α - and β -cyclodextrin inclusion complexes.
- Dekker, T.G.; Fourie, T.G.; Matthee, E.; Snyckers, F.O.; Ammann, W. 1987
South African Journal of Chemistry 40 (1) 74-76
Studies of South African medicinal plants. Part 4. Jaharin, a new daphnane diterpene with antimicrobial properties from *Jatropha zeyheri*.
- Derome, A.E. 1987
"Modern NMR techniques for chemistry research."
Organic Chemistry Series, Vol. 6. Ed. Baldwin, J.E.
Pergamon Press, Oxford.
- Domon, B.; Hostettmann, M.; Hostettmann, K. 1982 (a)
J. Chromatography 246 133-135
Droplet counter-current chromatography with non-aqueous solvent systems.
- Doman, B.; Hostettmann, K.; Kovacevic, K.; Prelog, V. 1982 (b)
J. Chromatography 250 149-151
Separation of the enantiomers of (+-)-norephedrine by rotation locular counter-current chromatography.
- Eight Peak Index of Mass Spectra, 2nd Ed. 1974
Mass Spectrometry Data Centre.
AWRE, Aldermaston, Reading.
- Elles, R.; Sutherland, I.A. 1980
Biochemical Society Transactions 8 173
Purification of specific restriction fragments of human deoxyribonucleic acid by using countercurrent chromatography.
- Evans, F.J.; Edwards, M.C. 1987
Bot. J. Linn. Soc. 94 (1/2) 231-246
Activity correlations in the phorbol ester series.
- Evans, F.J.; Kinghorn, A.D. 1977
Bot. J. Linn. Soc. 74 23-35
A comparative phytochemical study of the diterpenes of the genera *Euphorbia* and *Elaeophorbium* (Euphorbiaceae).
- Evans, F.J. 1986
"Phorbol: Its esters and derivatives."
Naturally Occurring Phorbol Esters. Ed. Evans, F.J. pg 171-216
CRC Press, Boca Raton, Florida.
- Evans, F.J.; Soper, C.J. 1978
Lloydia 41 (3) 193-233
The tiglane, daphnane and ingenane diterpenes, their chemistry, distribution and biological activities. A review.
- Evans, F.J.; Taylor, S.E. 1983
Progress in the chemistry of organic natural products 44 1-99
Pro-inflammatory, tumour-promoting and anti-tumour diterpenes of the plant families Euphorbiaceae and Thymelaeaceae.

- Fang, X.; Nanayakkara, N.P.O.; Phoebe, C.H.; Pezzuto, J.M.; Kinghorn, A.D.; Farnsworth, N.R. 1985
Planta Medica 1985 346-347
Plant anticancer agents XXXVII. Constituents of *Amanoa oblongifolia*.
- Gago, F.; Alvarez-Builla, J.; Elguero, J. 1986
J. Chromatography 360 247-251
Use of droplet counter-current chromatography in log *P* determinations.
- Govindachari, T.R.; Viswanathan, N.; Pai, B.R.; Rao, R.; Srinivasan, M. 1967
Tetrahedron 23 1901-1910
Triterpenes of *Calophyllum inophyllum* Linn.
- Gunasekera, S.P.; Sultanbawa, M.U.S. 1977
J. Chem. Soc. Perkin Trans. I 418-423
Chemical investigation of Ceylonese plants. Part 23. Extractives of *Hydnocarpus octandra* Thw. (Flacourtiaceae); Isolation and characterisation of six new triterpenoids.
- Hall, H.S.; Knight, S.R. 1946
"Higher Algebra".
Macmillan, London.
- Hanke, F.J.; Kubo, I. 1987
LC-GC 5 (3) 248-253
Recent examples of the use of droplet countercurrent chromatography in the chemistry of natural products.
- Hartwell, J.L. 1969
Lloydia 32 153-205
Plants used against cancer.
- Hecker, E. 1968
Cancer Research 28 2338-2349
Cocarcinogenic principles from the seed oil of *Croton tiglium* and from other Euphorbiaceae.
- Hecker, E. 1971
Cocarcinogens from Euphorbiaceae and Thymelaeaceae.
"Pharmacognosy and phytochemistry"
Ed. Wagner, H.; Horhammer, L.
Springer-Verlag, Berlin.
- Hecker, E. 1977
Pure and applied chemistry 49 1423-1431
New toxic and cocarcinogenic diterpene esters from Euphorbiaceae and Thymelaeaceae.
- Hecker, E. 1981
J. Cancer Res. Clin. Oncol. 99 103-124
Cocarcinogenesis and tumor promoters of the diterpene ester type as possible carcinogenic risk factors.

- Hecker, E. 1984
Acta Pharmacol. Toxicol. Suppl. 55 (2) 145-164
Co-carcinogens of the initiation- (or tumor) promoter type as environmental risk factors of cancer in man, experimental analysis of an etiologic model situation of life style cancer and current problems of assessment of cancer risk in multifactorial carcinogenesis.
- Hecker, E.; Adolf, W.; Hergenbahn, M.; Schmidt, R.; Sorg, B. 1984
"Irritant diterpene ester promoters of mouse skin: Contributions to etiologies of environmental cancer and to biochemical mechanisms of carcinogenesis."
Cellular Interactions by Environmental Tumor Promoters. pg 3-36.
Ed. Fujiki, H. et al
Japan Sci. Soc. Press, Tokyo
- Hecker, E.; Schmidt, R. 1974
Prog. Chem. Org. Nat. Prod. 31 377-467
Phorbol esters- the irritants and cocarcinogens of *Croton tiglium* L.
- Hermans-Lokkerbol, A; Verpoorte, R. 1986
Planta Medica 299-302
Droplet counter-current chromatography of alkaloids. The influence of pH-gradients and ion-pair formation on the retention of alkaloids.
- Hirata, Y. 1987
Bioact. Mol. 1987 (2) 181-205
The active principles of the Euphorbiaceae and Thymelaeaceae.
- Holmes, S. 1987
Royal Botanic Gardens, Kew, Richmond, Surrey.
Personal Communication.
- Hostettmann, K. 1980 (a)
Planta Medica 39 1-18
Droplet counter-current chromatography and its application to the preparative scale separation of natural products.
- Hostettmann, K. 1980 (b)
Chem. Rundsch., 33 (47) 9
Droplet counter-current chromatography (DCCC).
- Hostettmann, K. 1981
Chem. Labor Betr., 32 (5) 211-212
Droplet countercurrent chromatography.
- Hostettmann, K. 1983
Adv. Chromatogr. 21 165-168
Droplet counter-current chromatography.
- Hostettmann, K.; Appolonia, C.; Domon, B.; Hostettmann, M. 1984 (a)
J. Liquid Chromatography 7 (2) 231-242
Droplet countercurrent chromatography- new applications in natural products chemistry.
- Hostettmann, K.; Hostettmann, M.; Marston, A. 1984 (b)
Natural Products Reports 1 (5) 471-481
Isolation of natural products by droplet counter-current chromatography and related methods.

- Hostettmann, K.; Hostettmann, M.; Marston, A. 1986
"Preparative chromatography techniques. Applications in natural product isolation."
SpringerVerlag, Heidelberg.
- Hostettmann, K.; Hostettmann-Kaldas, M.; Sticher, O. 1979
J. Chromatography 186 529-534
Application of droplet counter-current chromatography to the isolation of natural products.
- Hui, W-H.; Ko, P.D.S.; Lee, Y-C.; Li, M-M.; Arthur, H.R. 1975
Phytochemistry 14 (11) 1063-1066
Triterpenes from ten *Lithocarpus* species of Hong Kong.
- Hui, W-H.; Li, M-M. 1976
Phytochemistry 15 (3) 427-429
Triterpenoids of the stems of six *Castanopsis* species of Hong Kong.
- Ito, Y. 1984
Advances in Chromatography 24 181-226
Development of high speed countercurrent chromatography.
- Ito, Y. 1986a
Trends in Analytical Chemistry 5 (6) 142-147
Trends in countercurrent chromatography.
- Ito, Y. 1986b
Critical reviews in analytical chemistry 17 (1) 65-143
High-speed countercurrent chromatography.
- Ito, Y.; Weinstein, M.; Aoki, I.; Harada, R.; Kimura, E.; Nunogaki, K. 1966
Nature 212 985-987
The coil planet centrifuge.
- Ito, Y.; Bowman, R.L. 1970
Science 167 281-283
Countercurrent chromatography: liquid-liquid partition chromatography without solid support.
- Ito, Y.; Bowman, R.L. 1971
Anal. Chem. 43 (13) 69a-75a
Countercurrent chromatography.
- Ito, Y.; Conway, W.D. 1984
Analytical Chemistry 56 (4) 534A-552A
Development of countercurrent chromatography.
- Jacobsen, H. 1977
"Lexicon of succulent plants." 2nd Ed.
Blandford Press, Poole, Dorset.
- Kemp, W. 1986
"NMR in chemistry. A multinuclear introduction."
Macmillan, London.

- Kinghorn, A.D. 1979
Toxic plants 6 137-159
Cocarcinogenic irritant Euphorbiaceae.
- Kinghorn, A.D. 1980
J. Pharm. Sci. 69 (12) 1446-1447
Major skin-irritant principle from *Synadenium grantii*.
- Kinsella, A.R. 1986
"Multi-stage carcinogenesis and the biological effect of tumour promoters."
Naturally Occuring Phorbol Esters. Ed. Evans, F.J. pg 33-61
C.R.C. Press, Boca Raton, Florida.
- Kinsella, A.R. 1987
Bot. J. Linn. Soc. 94 (1/2) 265-281
A review of the evidence from *in vitro* and *in vivo* studies for a role for phorbol ester tumour promoters from the Euphorbiales in the selection and clonal expansion of specific cell populations.
- Keulemans, A.I.M. 1957
"Gas chromatography"
Reinhold Publishing, New York.
- Knapp, A. 1985
Pharm. Unserer Zeit. 14 (3) 77-84
Tropfchengegenstromchromatographie und verwandte verfahren.
- Kokwaro, J.O. 1976
"Medicinal plants of East Africa."
East Africa Literature Bureau, Nairobi.
- Kolobow, T.; Ito, Y.; Mychkovsky, I.; Peters, P.; Morabito, J. 1985
J. Liquid Chromatogr. 8 (12), 2173-2193
Monolithic integrated flow circuit (MIFC): A new column design for countercurrent chromatography.
- Kulshreshtha, D.K.; Rastogi, R.R. 1971
Phytochemistry 10 (11) 2831-2832
Chemical constituents of *Quercus lanceaefolia*.
- Kumar, N.S.; Muthukuda, P.M.; Balasubramaniam, S. 1985
Phytochemistry 24 (10) 2454-2455
Triterpenes from *Euonymus revolutus*.
- Lim, T.K.; Soepadmo, E. 1984
Singapore Medical Journal 25 (6) 412-419
Eye injury from plant sap of *Pedilanthus tithymaloides* Poit.
- Lin, L.-J.; Kinghorn, A.D. 1983a
J. Agric. Food Chem. 31 396-400
Three new ingenane derivatives from the latex of *Euphorbia canariensis* L.
- Lin, L.-J.; Marshall, G.T.; Kinghorn, A.D. 1983b
J. Natural Products 46 (5) 723-731
The dermatitis-producing constituents of *Euphorbia hermentiana* latex.

- Lin, L.-J.; Kinghorn, A.D. 1983c
Phytochemistry 22 (12) 2795-2799
8-Methoxyingol esters from the latex of *Euphorbia hermentiana*.
- Mahlberg, P.G.; Davis, D.G.; Galitz, D.S.; Manners, G.D. 1987
Botanical Journal of the Linnean Society 1/2 165-180
Laticifers and the classification of *Euphorbia*: the chemotaxonomy of *Euphorbia esula* L.
- Mandava, N.B.; Ito, Y.; Conway, W.D. 1982
American Laboratory 14 (10) 62-78
Part One. Countercurrent chromatography: Historical development and early instrumentation.
- Mandava, N.B.; Ito, Y.; Conway, W.D. 1982
American Laboratory 14 (11) 48-57
Part Two. Countercurrent chromatography: Recent instrumentation and applications.
- Marshall, G.T.; Kinghorn, A.D. 1981
J. Chromatography 206 421-424
Isolation of phorbol and 4 phorbol from croton oil by droplet counter-current chromatography
- Martin, A.J.P.; Synge, R.L.M. 1941
Biochem. J. 35 1358-1368
A new form of chromatogram employing two liquid phases.
1. A theory of chromatography
2. Application to the micro-determination of the higher monoamino-acids in proteins.
- Martin, D.G.; Biles, C.; Peltonen, R.E. 1986
International Laboratory December 1986 18-30
Countercurrent chromatography in the fractionation of natural products.
- Martin, V.G.; Barford, R.A.; Eddy, C.R.; Rothbart, H.L. 1969
"Computer programs for countercurrent distribution"
U.S. Department of Agriculture, Philadelphia, Pennsylvania.
- Metzger, V.G.; Barford, R.A.; Rothbart, H.L. 1973
Separation Science 8 (2) 143-160
Chromatography and countercurrent distribution: Features of the optimum phase-volume ratio.
- Mitchell, J.; Rook, A. 1979
"Botanical Dermatology"
Greengrass, Vancouver.
- Morris, G.A. 1986
Magnetic Resonance in Chemistry 24 371-403
Modern NMR techniques for structure elucidation.
- Nishizawa, H.; Abe, Y. 1986
Analytical Sciences 2 (1) 97-98
A new apparatus for countercurrent chromatography: Liquid particle chromatography.

- Nozaki, H.; Suzuki, H.; Hirayama, T.; Kasai, R.; Wu, R-Y.; Lee, K-H. 1986
Phytochemistry 25 (2) 479-485
Antitumour triterpenes of *Maytenus diversifolia*.
- Orth 1981
Chem.-Anlagen Verfahren, (3) 128 130
Droplet counter-current chromatography.
- Oya, S.; Snyder, J.K. 1986
J. Chromatogr. 370 333-338
Chiral resolution of a carboxylic acid using droplet counter-current chromatography.
- Palmer, E.; Pitman, N.; Codd, L.E.W. 1972
"Trees of southern Africa." Vol. 2.
A.A. Balkema, Cape Town.
- Patra, A.; Chaudhuri, S.K. 1987
Mag. Reson. Chem. 25 95-100
Assignment of carbon-13 nuclear magnetic resonance spectra of some friedelanes.
- Pegel, K.H.; Piacenza, L.P.L.; Phillips, L.; Waight, E.S. 1971
Chemical Communications 1346-1347
ent-3 β -Hydroxy-15(16)-ene-2,12-dione from *Androstachys johnsonii* Prain (Euphorbiaceae).
- Piacenza, L.P.L.; Pegel, K.H.; Phillips, L.; Waight, E.S. 1979
J. Chem. Soc. Perkin Trans. I 1005-1012
Beyerane diterpenes: Structure and reactivity of the α -ketol *ent*-3 β -hydroxybeyer-15-ene-2,12-dione, its corresponding diosphenol and synthesis of the isomeric α -ketol acetates.
- Piacenza, L.P.; Pegel, K.H.; Laing, M.; Waight, E.S.; Weeks, C.M.; Gorst-Allman, C.P. 1985
J. Chem. Soc. Perkin Trans. I 703-709
A new atisane diterpene: *ent*-16 α -hydroxyatis-13-en-3-one from *Androstachys johnsonii* Prain.
- Pieters, L.A.; Vlietinck, A.J. 1986
Planta Medica 52 (6) 465-468
Rotational locular countercurrent chromatography and quantitative ¹H-nuclear magnetic resonance spectroscopy of the phorbol ester constituents of croton oil.
- Radcliffe-Smith, A. 1986
"A review of the family Euphorbiaceae"
Naturally occurring phorbol esters. Ed. F.J. Evans.
CRC Press, Boca Raton, Florida.
- Radcliffe-Smith, A. 1987
Bot. J. Linn. Soc. 94 (1/2) 47-66
Segregate families from the Euphorbiaceae.
- Rizk, A-F.M.; El-Missiry, M.M. 1986
"Non-diterpenoid constituents of Euphorbiaceae and Thymelaeaceae."
in Naturally Occuring Phorbol Esters. Ed Evans, F.J.
C.R.C. Press, Boca Raton, Florida.

- Rizk, A. F-M. 1987
Botanical Journal of the Linnean Society 1/2 293-326
"The chemical constituents and economic plants of the Euphorbiaceae."
- Rizvi, S.H.; Shoeb, A.; Kapil, R.S.; Popli, S.P. 1980
Experientia 36 146-147
Antidesmanol- a new pentacyclic triterpenoid from *Antidesma menasu* Miq. ex Tul.
- Roberts, H.B.; McClure, T.J.; Ritchie, E.; Taylor, W.C.; Freeman, P.W. 1975
Australian Veterinary Journal 51 325-326
The isolation and structure of the toxin of *Pimelea simplex* responsible for St. George disease of cattle.
- Rook. A.J. 1965
Brit. J. Dermatol. 77 (5) 284
An unrecorded irritant plant - *Synadenium grantii*. Skin sensitivity in man.
- Rothbart, H.L.; Barford, R.A. 1982
Countercurrent distribution.
"Treatise on analytical chemistry. Part 1: Theory and practice"
2nd Edition. Ed. P.J. Elving
John Wiley and Sons, New York.
- Rudall, P.J. 1987
Botanical Journal of the Linnean Society 1/2 143-163
Laticifers in Euphorbiaceae - a conspectus.
- Sakata, K.; Kawazu, K.; Mitsui, T.; Masaki, N. 1971
Tetrahedron Lett. (16) 1141-1144
Structure and stereochemistry of huratoxin, a piscicidal constituent of *Hura crepitans*.
- Sanders, J.K.M.; Hunter, B.K. 1987
"Modern NMR spectroscopy. A guide for chemists."
Oxford University Press, Oxford.
- Schmidt, R.J. 1987
Welsh School of Pharmacy, U.W.I.S.T.; Cardiff.
Personal communication.
- Schmidt, R.J.; Evans, F.J. 1977
Lloydia 40 (3) 225-229
The succulent Euphorbias of Nigeria. II. Aliphatic diterpene esters of the latices of *E. poisonii* Pax. and *E. unispina* N.E. Br.
- Schultes, R.E. 1987
Botanical Journal of the Linnean Society 1/2 79-95
Members of Euphorbiaceae in primitive and advanced societies.
- Seip, E.H.; Ott, H.H.; Hecker, E. 1983
Planta Medica 49 100-203
Skin irritant and tumor promoting diterpene esters of the tiglane type from the chinese tallow tree (*Sapium sebiferum*).

- Shoolery, J.N. 1984
J. Natural Products 47 (2) 226-259
Recent developments in ^{13}C - and proton-NMR.
- Signer, R. 1952
Chimia 6 243
Eine einfache gegenstromextractionsapparatur.
- Sixma, F.J.L.; Wynberg, H. 1964
A Manual of Physical Methods in Organic Chemistry.
John Wiley and Sons, New York.
- Snyder, J.K.; Nakanishi, K.; Hostettmann, K.; Hostettmann, M. 1984
J. Liquid Chromatogr. 7 (2) 243-256
Applications of rotational locular countercurrent chromatography in natural products isolation.
- Spoerke, D.G.; Montanio, C.D.; Rumack, B.H. 1985
Vet. Hum. Toxicol. 28 (4) 283-284
Pediatric exposure to the houseplant *Synadenium grantii*.
- Sutherland, I.A.; Heywood-Waddington, D.; Ito, Y. 1987
J. Chromatography 384 197-207
Countercurrent chromatography. Applications to the separation of biopolymers, organelles and cells using either aqueous-organic or aqueous-aqueous phase systems.
- Sutherland, I.A.; Heywood-Waddington, D.; Peters, T.J. 1984
J. Liquid Chromatogr. 7 (2) 363-384
Toroidal coil countercurrent chromatography: A fast simple alternative to countercurrent distribution using aqueous two phase partition.
- Sutherland, I.A.; Sharpe, J.E.E. 1976
J. Chromatography 122 333-344
Countercurrent chromatography using a new coil planet centrifuge.
- Takeuchi, T.; Horikawa, R.; Tanimura, T. 1984
J. Chromatography 284 285-288
Complete resolution of DL-isoleucine by droplet counter-current chromatography.
- Tanimura, T.; Otsuka, H.; Ogihara, Y. 1975
Kagaku No Ryoiki 29 (12) 895-902
Droplet countercurrent chromatography.
- Tanimura, T.; Pisano, J.J.; Ito, Y.; Bowman, R.L. 1970
Science 169 54-56
Droplet countercurrent chromatography.
- Taylor, S.E.; Gafur, M.A.; Choudhury, A.K.; Evans, F.J. 1981
Phytochemistry 20 (12) 2749-2751
Nitrogen-containing phorbol derivatives of *Sapium indicum*.
- Upadhyay, R.R.; Ansarin, M.; Zarintan, M.H.; Shakui, P. 1976
Experientia 32 1196-7
Tumor promoting constituent of *Euphorbia serrata* L. latex.

- van Alstein, J.M.; Snyder, R.S.; Karr, L.J.; Harris, J.M. 1985
J. Liquid Chromatogr. 8 (12) 2293-2313
Cell separation with countercurrent chromatography and thin-layer
countercurrent distribution in aqueous two phase system.
- van der Heijden, R.; Hermans-Lokkerbol, A.; Verpoorte, R.; Baerheim
Svendsen. 1987
J. Chromatography 396 410-415
Pharmacognostical studies of *Tabernamontana* species.
XX. Ion-pair droplet counter-current chromatography of indole alkaloids
from suspension cultures.
- van Wyk, P. 1974
"Trees of the Kruger National Park" Vol. 2.
Purnell, Cape Town.
- Watt, J.M.; Breyer-Brandwijk, M.G. 1962
"Medicinal and poisonous plants of southern and eastern Africa." 2nd Ed.
Livingston, London.
- Webster, G.L. 1975
Taxon 24 (5/6) 593-601
Conspectus of a new classification of the Euphorbiaceae.
- Webster, G.L. 1987
Botanical Journal of the Linnean Society 1/2 3-46
The saga of the spurges: a review of classification and relationships in
the Euphorbiales.
- Willis, J.C. 1973
"A dictionary of the flowering plants and ferns." 8th Ed.
Revised by Airy Shaw, H.K.
Cambridge University Press, Cambridge.
- Wiriyachitra, P.; Hajiwangoh, H.; Boonton, P.; Adolf, W.; Opferkuch, H.J.;
Hecker, E. 1985
Planta Medica 1985 368-371
Investigations of medicinal plants of Euphorbiaceae and Thymelaeaceae
occurring and used in Thailand; II. Cryptic irritants of the diterpene
ester type from three *Excoecaria* species.
- Zechmeister, K.; Rohrl, M.; Brandle, F.; Hechtfisher, S.; Hoppe, W.;
Hecker, E.; Adolf, W.; Kubinyi, H. 1970
Tetrahedron Letters 3071-3073
Röntgenstrukturanalyse eines neuen makrozyklischen diterpenestes aus der
Springwolfsmilch (*Euphorbia lathyris* L.).
- Zeng, Y.; Miao, X.C.; Jaio, B.; Li, H.Y.; Ni, H.Y.; Yohei, I. 1984
Cancer Letters 23 53-59
Epstein-Barr Virus activation in Raji cells with ether extracts of soil
from different areas of China.